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FINAL REPORT
for the Project

**Behavioral and Physiological Effects of
Hindlimb Unloading in Rats**

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TABLE OF CONTENTS

OVERVIEW	3
INTRODUCTION	3
TREATMENT PARADIGMS	4
MOTOR RESPONSE EXPERIMENTS	4
<u>Behavioral Measures and Effects</u>	4
NEUROPHYSIOLOGICAL MEASURES	5
NEUROCHEMICAL EFFECTS	6
MORPHOLOGICAL EFFECTS	7
REFERENCES	8
APPENDIX A. Meeting Abstract	10
APPENDIX B. Bibliography and Reprints.....	13

Behavioral and Physiological Effects of Hindlimb Unloading in Rats

Overview

The overarching objective of this project was to identify changes in neural and biochemical systems of the central and peripheral nervous systems (the CNS and PNS) that are related to disruptions of functional motor responses, or motor control. The identification of neural and biochemical changes that are related to sensory-motor adaptation elicited as animals react to changes in the gravitational field was of particular interest. Thus, the major objective of this work was to study disruptions of motor responses that arise after (*sic.* due to) chronic exposure to altered gravity (G). To do this, parallel studies investigating changes in neural, sensory, and neuromuscular systems were conducted after animals (rats) experienced chronic exposure to conditions of altered-G. Conditions of altered-G included hyper-G produced by centrifugation, micro-G produced by orbital flight, and simulated micro-G produced by hind limb suspension. A second major interest was to examine the contribution of putative changes in sensory systems to disruptions of motor responses. To do this, motor responses and reflexes of rats were studied following chronic treatment with streptomycin sulfate (STP, an ototoxic chemical) to damage the vestibular hair cells.

Introduction

Chronic exposure to conditions of altered-G has profound effects on many physiological systems. Alterations in motor control also are well documented. All extant organisms have evolved under constant influence of the 1-G field of Earth, and deviations from this gravitational field have profound effects on motor control, spatial orientation, gaze control and equilibrium. In addition, perceptual problems and motion sickness often occur when individuals are subjected to new, or different, gravitational conditions (Daunton, 1996). Thus, both physiological and sensory-motor reactions to conditions of altered-G likely contribute to the processes of adaptation that occur during chronic exposure to either hyper-G or micro-G.

Changes in the morphology and physiology of sensory systems finely tuned to G-forces also may be important in adaptation to conditions of altered-G. Ross (1993) has shown that the number of synapses on gravity-sensitive hair cells changes following chronic exposure either to hyper-G or to micro-G. In addition, electrophysiological studies during and after space flight have shown changes in activity of otolithic afferents and otolith-related units in the vestibular nuclei (see Daunton, 1996), and vestibulo-spinal reflexes are altered during and following space flight (Watt et al., 1986; Reschke et al., 1986; Daunton, 1996). These effects suggest that sensory information from the vestibular hair cells, particularly the otoliths, may be critically involved in the processes of sensory-motor adaptation in conditions of altered-G.

The fundamental strategy of the research in this project was to conduct parallel studies of motor responses, neural structures, and physiology to further understanding of the mechanisms underlying sensory-motor adaptation. Studies of motor responses included examination of postural control, orientation and locomotion following chronic exposure to hyper-G, micro-G, and hind limb suspension (simulated micro-G) and, in some cases, after damaging vestibular hair cells with STP. Results of studies investigating motor responses (using air-righting, swimming,

locomotion and posture as measures) were compared with parallel studies of physiological (EMG), neurochemical (GABA immunoreactivity, neuropeptides), and morphological effects (neuromuscular junctions, dorsal root ganglia, spinal cord, synapses, glia) of the same treatments using similar paradigms.

Treatment Paradigms

Because of the technical complications of testing during chronic exposure to conditions of altered G, the basic paradigm is one in which changes induced by chronic exposure are studied in tests conducted after the exposure (e.g., after return to 1G following a period of exposure to hyper-G). This strategy is based on the hypothesis that this form of sensory-motor adaptation, like other types of adaptation, involves three stages of adaptation and re-adaptation involving (1) initiation of the processes, (2) consolidation of changes, and (3) maintenance of new status during which responses are stabilized and continued (Daunton, 1996). Disruption in a measure in an initial test conducted soon after removal from the chronic treatment provides an estimate of the magnitude of change that occurred during the exposure, and thus estimates the degree of adaptation that has occurred. Additional tests at varying durations after removal from the chronic treatment provide an estimate of the rate of change in systems that are readapting to normal conditions.

Conditions of altered-G were produced using hyper-G produced by centrifugation (CF), micro-G resulting from space flight (SF), or simulated micro-G using hind limb suspension (HLS). In all studies chronic exposure was conducted in conditions that permitted active movements of the animals during the treatment. Studies of altered-G were conducted using young, male Sprague-Dawley rats. Effects of damage to vestibular hair cells were conducted using young, male Long-Evans (pigmented) rats (see Meza et al., 1996).

Motor Response Experiments

Although effects of altered G have been documented in humans (e.g., Lackner, 1993) and fish (Rahmann et al., 1990), little information directly described the effects of altered G on motor responses in other species when this project began. Consequently, it was important to identify measures that would reliably reflect the processes of adaptation of motor systems to altered G. This work was organized around the hypothesis that this form of sensory-motor adaptation, like others, involves three stages of adaptation and re-adaptation involving (1) initiation of the processes, (2) consolidation of changes, and (3) maintenance of new status during which responses are stabilized and continued (Daunton, 1996). Behavioral testing was conducted to identify reliable measures and to evaluate the possible timing of these three stages of adaptation, or re-adaptation for each sensory-motor system assessed. These results then were used to relate stages of re-adaptation to morphological and neurochemical changes found in the nervous system.

Behavioral Measures and Effects

Two behavioral responses that are thought to be controlled importantly by input from the otolith organs, the air-righting reflex and orientation during swimming, show significant

Behavioral & Physiological Effects

disruption following exposure to hyper-G (Fox et al., 1992; 1998), and HLS (Fox et al., 1993). While both air-righting and swimming were disrupted after exposure to 2G, neither measure was disrupted in Rotation Control animals. Thus, both measures reflect the effects of hyper-G independent of the rotation component of centrifugation that is used to produce hyper-G. The recovery of normal air-righting is more rapid after 14 days of exposure to HLS than after 14 days of exposure to 2G (Fox et al., 1998).

Both air-righting and swimming are disrupted after treatment with STP (Meza et al., 1996). Abnormal swimming patterns consisted of vertical swimming with rolls, barrel rolling, corkscrew swimming and forward and backward looping. Partial recovery of swimming was observed in animals 8 weeks after STP treatment was discontinued, but vertical swimming with rolls remained in all animals after 8 weeks. These abnormal swimming patterns are consistent with effects seen in mice that are congenitally otolith-deficient and suggest that treatment with STP preferentially damages gravity-sensitive hair cells.

Effects of hyper-G and HLS on gait walking on a hard surface and limb movement during swimming are summarized in Fox et al. (1998). Inter-limb coordination is disrupted following both treatments, but recovers more rapidly following exposure to 2G (see Fox et al., 1992, 1993, 1994) than following exposure to HLS where effects last for several weeks (Fox, unpublished data). Effects of space flight and HLS are summarized in Fox et al. (1994). Six hours after returning from orbital flight rats walked with the back dorsiflexed, the hind quarters lower than normal, and with the tail dragging. This is in stark contrast to HLS rats which walk with the back straight or ventro-flexed relative to normal, the hind quarters elevated, and the tail held very high off the floor. Space flight rats also walked with extreme dorsiflexion (plantar extension) of the ankle that resulted in foot placement similar to that seen in rats of approximately 10 days of age.

Neurophysiological Measures

The early EMG response in hind leg muscles to sudden drop in the prone position (the Free-Fall Response, or FFR) is dependent on the vestibular system in humans (Greenwood & Hopkins, 1976), cats (Watt, 1976) and baboons (Lacour et al., 1978, 1979). Gruner (1989) demonstrated this response also occurs in the rat, but did not evaluate dependence of the response on the vestibular system. To evaluate the stimulus for this response in rats, pigmented rats were treated chronically with intramuscular injections of STP using the method of Meza et al. (1996) to damage otolith hair cells. These animals then were tested using the FFR method of Gruner. Following treatment with STP the amplitude of EMG responses elicited by free-fall was severely depressed in the lateral gastrocnemius muscle, but the amplitude of the EMG elicited by auditory stimulation (i.e., the auditory startle reflex) was unaffected (Fox et al., 1997, see Appendix A). These results suggest the FFR is elicited by stimulation of vestibular origin, presumably the otolith hair cells, in the rat as it is in other species.

To test whether the gain in the otolith portion of the vestibular system may be reduced by exposure to hyper-G, the effects of chronic exposure to hyper-G on the FFR were investigated using a parallel experiment. The EMG elicited by sudden fall occurred inconsistently and with greatly suppressed amplitude (Fox, et al., 1998). This effect is consistent with the disruption of

motor responses and with the work of Ross (1993) which suggests reduced gain may occur following hyper-G. Similar disruption has been shown during orbital flight (Watt et al., 1986) suggesting a reduction in vestibular influence on the motor system occurs during orbital flight (see Fox et al., 1998 for further discussion).

Neurochemical Effects

Radioimmunoassay techniques were used to investigate neurochemical effects possibly related to adaptation to altered-G. Neuropeptide levels were assessed in brainstem, cerebellum, hypothalamus, striatum, hippocampus, and cerebral cortex to screen for possible changes. Rats were exposed to 2G or to rotation only (Rotation Controls) for 14 days. Levels of thyrotropin-releasing hormone (TRH) were increased in brainstem and cerebellum, but no changes were observed in β -endorphin, cholecystokinin, met-enkephalin, somatostatin, or substance P in any areas of the CNS studied. In addition, levels of TRH were not significantly changed in areas other than brainstem and cerebellum, and TRH was not affected in animals exposed only to the rotational component of centrifugation. Thus, it appears that effects on TRH were elicited by changes in the gravitational component of centrifugation.

Increases in TRH only in areas of the brain known to be importantly involved with vestibular inputs and both voluntary and reflexive motor control. These results suggest that TRH may play a role in adaptation to altered-G as it does in adaptation following labyrinthectomy and in cerebellar and vestibular control of locomotion as seen in studies of ataxia.

Effects of HLS and CF on adaptation in the CNS also were examined by studying GABA-ergic neurons in rat somatosensory cortex. Following 14 days of HLS the number of GABA-immunoreactive cells was reduced in layers Va and Vb and GABA-containing terminals also were reduced in the same layers (see D'Amelio et al., 1996). GABA-containing terminals surrounding the soma and apical dendrites of pyramidal cells of layer Vb were particularly affected. Unloading of weight bearing by HLS may alter afferent input from intramuscular receptors to the CNS and elicit reorganization of hind limb muscle groups. The reduced immunoreactivity of GABA-ergic neurons and terminals may reflect changes in modulatory activity contributing to compensation for altered afferent stimulation due to the treatment.

To obtain improved quantification of immunoreactive terminal area we developed a light microscope image analysis system that can be applied to studies of this type (Wu et al., 1997). This technique is based on Fast Fourier Transform routines available in the NIH-image public domain software. The procedure provides an objective means of measurement of area by counting the total pixels occupied by immunoreactive terminals in sections, and minimizes the difficulties that arise from labeling intensity, size, shape and numerical density of terminals.

This procedure was used to assess effects of exposure to hyper-G for 14 days on GABA-immunoreactivity (GABA-IR) in rat somatosensory cortex (D'Amelio et al., 1998). The area of GABA-IR terminals apposed to the soma of pyramidal cells in cortical layer V was reduced following chronic exposure to hyper-G but was unaffected in rotational control rats. This reduction in GABA-IR of the terminal area around pyramidal neurons may reflect changes in cells that are involved in reprogramming of motor outputs to achieve effective movement control in the altered-G environment.

Morphological Effects

The suggestion that GABA immunoreactivity may be related to modulatory activity of GABA as part of the process of adaptation to altered-G is consistent with neuromuscular changes observed in space flight (D'Amelio et al., 1998). Study of the adductor longus (an antigravity muscle) after space flight revealed myofiber atrophy, segmental necrosis and regenerative myofibers which were immunoreactive to N-CAM. Neuromuscular junctions contained axon terminals with decreased or absent synaptic vesicles and vacant axonal spaces suggestive of axonal sprouting. These findings suggest that muscle regeneration and denervation and synaptic remodeling may occur at the level of the neuromuscular junction during space flight. Such changes could result in significant alterations of afferent feedback to the CNS and might elicit major remodeling of neuromuscular control systems, some of which may be in rat somatosensory cortex.

References

- Daunton, N. G. (1996). Adaptation of the vestibular system to microgravity. In M. J. Fregly and C.M. Blatteis (Eds.), Handbook of physiology, Section 4: Environmental Physiology Vol 1, Oxford University Press, Oxford, pp. 765-783.
- Fox, R. A., Corcoran, M., Daunton, N. G., & Morey-Holton, E. (1994). Effects of spaceflight and hindlimb suspension on the posture and gait of rats. In K. Tagachi, M. Igarashi, & S. Mori (Eds.) Vestibular and Neural Front. Amsterdam: Elsevier Science B.V.
- Fox, R. A., Daunton, N. G., & Corcoran, M. L. (1998). Study of adaptation to altered gravity through systems analysis of motor control. Advances in Space Research, 22(2), 245-253.
- Fox, R. A., Daunton, N., Corcoran, M., Wu, L. (1992). Time-course of behavioral adaptation to hyper-gravity. In M. Woolacott & F. Horak (Eds.), Posture and gait: Control mechanisms. University of Portland Books, Portland Oregon. pp. 178-181.
- Fox, R. A., Daunton, N. G., Corcoran, M. L., Wu, L. C., & D'Amelio, F. (1993). Tail suspension with and without hindlimb unloading affects neuromuscular function in the adult rat. Neuroscience Abstracts, 19(1), 147.
- Greenwood, R., & Hopkins, A. (1976). Muscle responses during sudden falls in man. Journal of Physiology, 254, 507.
- Gruner, J. A. (1989). Comparison of vestibular and auditory startle responses in the rat and cat. Journal of Neuroscience Methods, 27, 13.
- Lackner, J. R. (1993). Orientation and movement in unusual force environments. Psychological Science, 4(3), 134.
- Lacour, M., Xerri, C., & Hugon, M. (1978). Muscle responses and monosynaptic reflexes in falling monkey: Role of the vestibular system. Journal of Physiology, 74, 427.
- Lacour, M., Xerri, C., & Hugon, M. (1979). Compensation of postural reactions to fall in the vestibular neurotized monkey. Role of the remaining labyrinthine afferences. Experimental Brain Research, 39, 363.
- Meza, G., Bohne, B., Daunton, N., Fox, R., & Knox, J. (1996). Damage and recovery of otolithic function following streptomycin treatment in the rat. In (Eds.), New directions in vestibular research, 781, 666-669.
- Rahmann, H., Slenzka, K., Hilbig, R. (1990). Effect of hyper-gravity on the swimming behavior of aquatic vertebrates. In Fourth European Symposium on Life Sciences Research in Space, ESA SP-307, pp. 259-263.
- Reschke, M., Anderson, D., & Homick, J. (1986). Vestibulospinal response modifications as determined with the H-reflex during the Spacelab-1 Flight. Experimental Brain Research, 64, 367.
- Ross, M. D. (1993). Morphological changes in the rat vestibular system following weightlessness. Journal of Vestibular Research, 3, 241.

- Watt, D.G.D. (1976). Responses of cats to sudden falls: An otolith-originating reflex assisting landing. Journal of Neurophysiology, 39(2), 257.
- Watt, D.G.D., Money, K. E., & Tomi, L. M. (1976). M.I.T./Canadian vestibular experiments on the Spacelab-1 Mission: 3. Effects of prolonged weightlessness on human otolith-spinal reflex. Experimental Brain Research, 64, 308.

APPENDIX A

Reprint of Abstract submitted to the Meeting of the Society for Posture and Gait
in Paris, France, 1997.

EFFECTS OF STREPTOMYCIN-INDUCED HAIR CELL DAMAGE ON THE FREE-FALL REFLEX IN RATS

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Unexpected falls evoke electromyographic (EMG) responses in both flexor and extensor muscles of the legs in humans, monkeys, cats and rats. This EMG response, called the free-fall response (FFR; Gruner, 1989), is thought to play an important role in the production of appropriate muscle tone in preparation for landing at the end of a fall (Watt, 1976). The FFR is an otolith-spinal reflex in humans (Greenwood & Hopkins, 1976) and cats (Watt, 1976) and, although it also appears in the rat (Gruner, 1989), the specific origin of the response has not been determined for this species. In this experiment the impact of damage to the otolithic hair cells on the FFR was investigated to evaluate whether this response also is an otolith-mediated reflex in the rat.

It is well known that streptomycin (STP) is an ototoxic drug that affects vestibular and auditory hair cells. Chronic treatment of the pigmented rat with STP disrupts motor behaviors directly related to otolithic function, leaving auditory and semicircular canal functions intact (Meza *et al.*, 1996). Abnormal air-righting and swimming (vertical barrel rolls, corkscrew turns and forward and backward loops) occur in rats treated with STP, but there is no alteration of either auditory evoked potentials or post-rotatory nystagmus. These disruptions of air-righting and swimming are similar to those observed in mice and rats with congenital otoconial deficiencies. Histological examination of vestibular hair cells of rats treated with STP reveal fused stereocilia and pyknotic nuclei in the utricular macula while cells in the cristae and organ of Corti appear normal. These findings suggest that chronic treatment of the pigmented rat with STP may selectively damage hair cells in the linear-acceleration-sensitive otolith organs. Consequently, STP ototoxicity was used to examine the impact of selective disruption of otolithic function on the FFR in rats.

Procedures

Adult male Long-Evans rats were chronically treated with intramuscular injections (Pfizer, 400 mg/kg per day) of STP in the forelegs for up to 35 days. Control rats were injected with an equivalent volume of sterile saline. Air-righting and swimming were tested at 21 and 35 days of treatment to assess the extent of disruption of otolith-mediated behavior. After 35 days of treatment, bipolar EMG electrodes were implanted in the lateral gastrocnemius (LG) and tibialis anterior (TA) muscles to measure responses to a loud noise (startle) and to sudden falls (otolith-spinal reflex, FFR).

Results

The auditory startle reflex remained normal in animals treated with STP implying that cochlear hair cells were not damaged. However, magnitude of the FFR decreased in both the TA and LG following treatment with STP (see Fig. 1), and the latency of the FFR became more variable. In some rats, low amplitude, periodic EMG spikes reflecting apparent desynchronization of the response occurred for up to 40 ms after stimulation by the sudden drop (see Fig. 2). This low amplitude activity occurred on trials with and without a clearly defined FFR. These effects were found when air-righting and swimming were only minimally disrupted, suggesting that even minimal damage to hair cells of the otolith organs can disrupt this response in the rat.

Reduction of the gain and disruption of synchronization in the FFR after hair cells presumably are damaged by STP suggest that the FFR is an otolith-spinal reflex in the rat. Additional evidence indicating that the FFR is mediated by otolithic inputs is provided by the finding that FFR gain is reduced in rats subjected to hyper-gravity induced by centrifugation (Fox *et al.*, in press). However, the desynchronized EMG activity seen following the FFR was not observed after chronic exposure to hyper-gravity. Thus, a reduction in gain of the FFR can occur without

disruptions of synchronization following the FFR. In vitro studies using direct application of STP have shown rapid changes in membrane properties of vestibular hair cells and effects on calcium channels in muscle. The contribution of these STP-induced alterations to changes in the gain and synchronization of the EMG following the FFR will be examined in future studies.

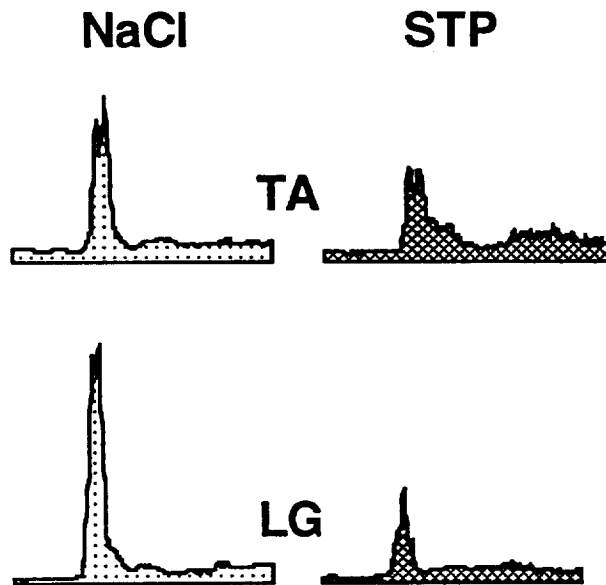


Fig. 1. Average EMG activity during the FFR in Control rats ($n=3$) injected with NaCl and in rats ($n=3$) treated with STP.

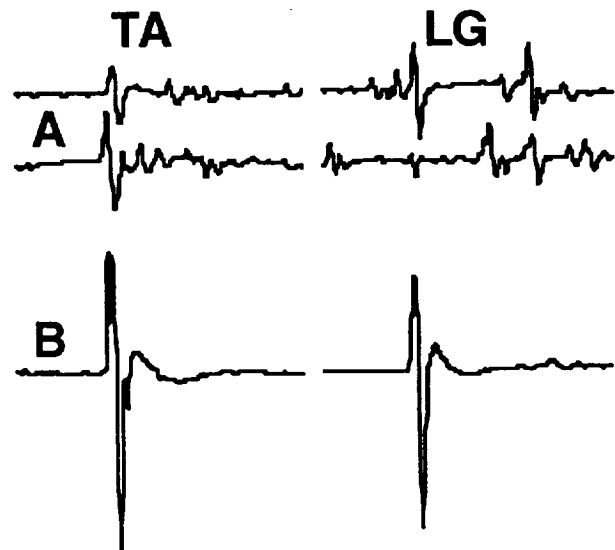


Fig. 2. (A) Raw EMG traces illustrating desynchronization of the EMG following free fall in a rat treated with STP. (B) The typically rapid return to baseline EMG activity following the FFR in a Control rat. All traces are displayed at the same amplification.

References

- Fox, R. A., N. G. Daunton, , & M. Corcoran, *Advances in Space Research*, in press.
 Greenwood, R., & A. Hopkins, *J. Physiol.*, 254, 507, 1976.
 Gruner, J. A., *J. Neurosci. Methods*, 27, 13, 1989.
 Meza, G., B. Bohne, N. Daunton, R. Fox, & J. Knox, *Ann. New York Academy Sci.*, 781, 666, 1996.
 Watt, D. G. D., *J. Neurophys.*, 39(2), 257, 1976.

APPENDIX B

Listing of Reprints followed by Reprints

- D'Amelio, F., Fox, R. A., Wu, L. C., Daunton, N. G., & Corcoran, M. L. (1998). Effects of microgravity on muscle and cerebral cortex: A suggested interaction. Advances in Space Research, 22(2), 235-244.
- D'Amelio, F., Wu, L. C., Fox, R. A., & Daunton, N. G. (1995). Quantitative changes of GABA-immunoreactive cells in the hindlimb representation of the rat somatosensory cortex after 14-day hindlimb unloading by tail suspension. Neuroscience Abstracts, 21, 1901.
- D'Amelio, F., Wu, L. C., Fox, R. A., & Daunton, N. G. (1996). Quantitative changes of GABA-immunoreactive cells in the hindlimb representation of the rat somatosensory cortex after 14-day hindlimb unloading by tail suspension. Journal of Neuroscience Research, 44, 532-539.
- D'Amelio, F., Wu, L. C., Fox, R. A., Daunton, N. G., Corcoran, M. L., & Polyakov, I. (1998). Hypergravity exposure decreases γ -aminobutyric acid immunoreactivity in axon terminals contacting pyramidal cells in the rat somatosensory cortex: A quantitative immunocytochemical image analysis. Journal of Neuroscience research, 53, 135-142.
- Daunton, N. G., Tang, F., Corcoran, M. L., Fox, R. A., & Man, S. Y. (1998). Chronic exposure to hypergravity affects thyrotropin-releasing hormone levels in rat brainstem and cerebellum. Biological Signals and Receptors, 7, 337-344.
- Fox, R. A., Corcoran, M. L., Daunton, N. G., & Morey-Holton, E. (1994). Effects of spaceflight and hindlimb suspension on the posture and gait of rats. In K. Taguchi, M. Igarashi, & S. Mori (Eds.) Vestibular and neural front, Elsevier Science B.V., Amsterdam.
- Fox, R., Corcoran, M., Taber, P., & Wu, L. (1997). Suppression of otolith-spinal reflex by chronic hypergravity exposure and streptomycin treatment. Neuroscience Abstracts, 23(1), 754.
- Fox, R. A., Daunton, N., Corcoran, M., Wu, L. (1992). Time-course of behavioral adaptation to hyper-gravity. In M. Woolacott & F. Horak (Eds.), Posture and gait: Control mechanisms. University of Portland Books, Portland Oregon. pp. 178-181.
- Fox, R. A., Daunton, N. G., Corcoran, M. L., Wu, L. C., & D'Amelio, F. (1993). Tail suspension with and without hindlimb unloading affects neuromuscular function in the adult rat. Neuroscience Abstracts, 19(1), 147.
- Fox, R. A., Daunton, N. G., & Corcoran, M. L. (1998). Study of adaptation to altered gravity through systems analysis of motor control. Advances in Space Research, 22(2), 245-253.
- Fox, R. A., Knox, J., Skinner, J., & Spomer, M. (1995). Functional deafferentation of knee joint afferents produces leg extension and knuckle walking in rats. Neuroscience Abstracts, 21, 420.

- Fox, R. A., Moreno, C., & Knox, J. (1996). Effects of section and spontaneous reattachment of the soleus muscle on the gait of the adult rat. Neuroscience Abstracts, 22(3), 2042.
- Meza, G., Bohne, B., Daunton, N., Fox, R., & Knox, J. (1996). Damage and recovery of otolithic function following streptomycin treatment in the rat. In (Eds.), New directions in vestibular research, 781, 666-669.
- Polyakov, I. V., Kaufman, G. D., Daunton, N. G., Fox, R. A., & Perachio, A. A. (1997). The effect of galvanic translabyrinthine utricular stimulation on FOS expression in the gerbil brain and spinal cord. Neuroscience Abstracts, 23(2), 1291.
- Wu, L. C., D'Amelio, F., Fox, R. A., Polyakov, I., & Daunton, N. G. (1997). Light microscopic image analysis system to quantify immunoreactive terminal area apposed to nerve cells. Journal of Neuroscience Methods, 74, 89-96.
- Wu, L. C., D'Amelio, F., Polyakov, I., Daunton, N. G., & Fox, R. A. (1996). Affordable image analysis system to quantify immunoreactive terminals in the somatosensory cortex. Cell Vision (abstract), 3(3), 249.

Paper presented at Scientific Meeting abstract is in Appendix A.

- Taber, P., Fox, R. A., Duran, M. L., Daunton, N. G., Meza, G. and Manzano, D. (1997). Effects of streptomycin-induced hair cell damage on the free-fall reflex in rats. Paper presented at the Meeting of the International Society for Posture and Gait, Paris, France, July.



EFFECTS OF MICROGRAVITY ON MUSCLE AND CEREBRAL CORTEX: A SUGGESTED INTERACTION

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ABSTRACT

The "slow" antigravity muscle adductor longus was studied in rats after 14 days of spaceflight (SF). The techniques employed included standard methods for light microscopy, neural cell adhesion molecule (N-CAM) immunocytochemistry and electron microscopy. Light and electron microscopy revealed myofiber atrophy, segmental necrosis and regenerative myofibers. Regenerative myofibers were N-CAM immunoreactive (N-CAM-IR). The neuromuscular junctions showed axon terminals with a decrease or absence of synaptic vesicles, degenerative changes, vacant axonal spaces and changes suggestive of axonal sprouting. No alterations of muscle spindles was seen either by light or electron microscopy. These observations suggest that muscle regeneration and denervation and synaptic remodeling at the level of the neuromuscular junction may take place during spaceflight.

In a separate study, GABA immunoreactivity (GABA-IR) was evaluated at the level of the hindlimb representation of the rat somatosensory cortex after 14 days of hindlimb unloading by tail suspension ("simulated" microgravity). A reduction in number of GABA-immunoreactive cells with respect to the control animals was observed in layer Va and Vb. GABA-IR terminals were also reduced in the same layers, particularly those terminals surrounding the soma and apical dendrites of pyramidal cells in layer Vb. On the basis of previous morphological and behavioral studies of the neuromuscular system after spaceflight and hindlimb suspension it is suggested that after limb unloading there are alterations of afferent signaling and feedback information from intramuscular receptors to the cerebral cortex due to modifications in the reflex organization of hindlimb muscle groups. We propose that the changes observed in GABA immunoreactivity of cells and terminals is an expression of changes in their modulatory activity to compensate for the alterations in the afferent information.

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INTRODUCTION

The first section of this report will place emphasis upon some particular responses to weightlessness observed in the adductor longus muscle of rats flown in the Soviet COSMOS flight 2044, namely, 1) muscle fiber injury, 2) regenerative phenomena, and 3) alterations of the neuromuscular junctions. In previous studies, investigations carried out upon different muscles after both flight and ground-based (mostly hindlimb suspension) experiments have provided information on the effects of microgravity and "simulated" microgravity upon morphology, metabolic properties, histochemistry and electrophysiology (see Edgerton and Roy, for review, 1994). Through these studies we have learned that "slow" muscles, mostly composed of type I fibers (e.g., soleus, adductor longus), carry the burden of the changes while "fast" muscles, mostly composed of type II fibers (e.g., tibialis anterior) are relatively unaffected.

The second section of this report will deal with the possible consequences that limb unloading may have upon those areas of the central nervous system related to sensory inputs from muscles. Our assumption—based on our current behavioral and morphological studies (D'Amelio *et al.*, 1987; D'Amelio and Daunton, 1992; Fox *et al.*, 1993, 1994)—was that muscle atrophy produced by limb unloading could modify sensory inputs arising from muscle receptors to the cerebral cortex. We focused our analysis on the behavior of GABAergic neurons of the hindlimb representation of the somatosensory cortex since numerous lines of research have demonstrated modifications in the level of GABA-IR or glutamic acid decarboxylase (GAD) immunoreactivity in cortical interneurons when sensory activity is altered by surgical manipulation (Hendry and Jones, 1986; Warren *et al.*, 1989; Akhtar and Land, 1991; see also Jones, 1990).

MATERIAL

Muscle Study

Wistar-derived male rats (SPF) from the Institute of Endocrinology, Bratislava, Czechoslovakia, aged approximately 3.5 months and weighing on average 180 grams at launch, were used in this experiment. Five animals per group (1 flight group and 3 control groups) were employed. The animals were not subjected to any type of invasive procedure. The flight animals remained for 14 days exposed to the space environment. Animal handling, launching details, as well as the procedures employed on muscle tissue have been described elsewhere (D'Amelio and Daunton, 1992).

Cerebral Cortex Study

Hindlimb unloading by tail suspension (HLS) to simulate some of the effects of weightlessness on muscles observed following spaceflight (SF) (see Ilyin and Oganov, 1989; Thomason and Booth, 1990; Edgerton and Roy, 1994, for reviews) was employed for this study. Six Sprague-Dawley rats (200–250 g) were employed. Three served as controls and three were suspended (HLS) by the tail for 14 days. The hindlimb representation of the somatosensory cortex was identified in Nissl-stained slides by the prominent aggregation of granular cells in layer IV. GABA-IR cell counts were done on pair of sections (control and experimental) on the same slides. Particulars of suspension procedure, perfusion of animals, immunostaining and methodology for quantitative analysis of GABAergic cells have been published elsewhere (D'Amelio *et al.*, 1996).

RESULTS

Muscle Study

The main alterations observed in all the flight animals, and not in any of the control animals, were myofiber atrophy, segmental necrosis (frequently accompanied by extensive cellular infiltration composed of macrophages, polymorphonuclear leukocytes and mononuclear cells) (Figure 1) and regenerating myofibers that were immunoreactive to N-CAM (Figure 2). For the quantitative assessment of myofiber atrophy Z band length was measured to approximate myofiber diameter in electron microphotographs. In the flight animals Z band length ranged from 1,460 Å to 2,600 Å with a mean of 2,095 Å while in the control animals the range was from 3,100 Å to 3,500 Å with a mean of 3,109 Å ($F(1,6) = 8.55$, $p = .0265$).

The most salient changes of the neuromuscular junctions were: absence of synaptic vesicles with replacement by microtubules and neurofilaments, interposition of Schwann cell processes between pre- and postsynaptic membranes, "unemployed" axonal spaces with shallow primary clefts, complete degeneration of axon terminals, and axonal sprouting (Figures 3 and 4). Of the 40 neuromuscular junctions from flight animals 24 (89%) showed one or more of these changes. In the 38 neuromuscular junctions from control animals only 11% showed one or more of these changes ($X^2 = 23.38$; $p < .0001$). No alterations of muscle receptors (i.e., muscle spindles) was seen in our preparations.

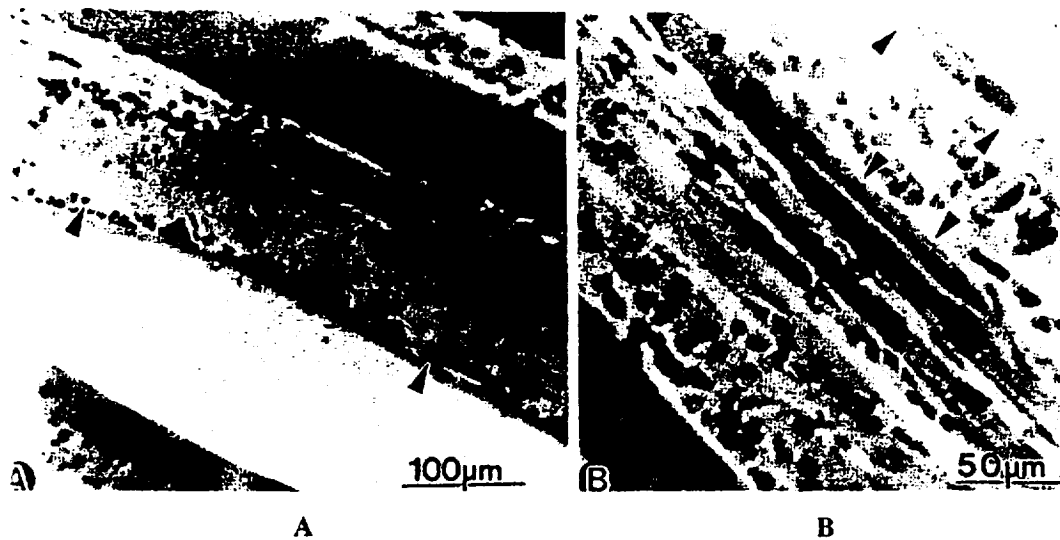


Fig. 1. Flight animals. In (A), longitudinal sections show segmental necrosis of myofibers (arrowheads) accompanied by inflammatory cellular infiltration. In (B), atrophic fibers (arrowheads), edema and cellular infiltrates mainly composed of histiocytes and polymorphonuclear leukocytes. From D'Amelio and Danton (1992), with permission from the publisher.

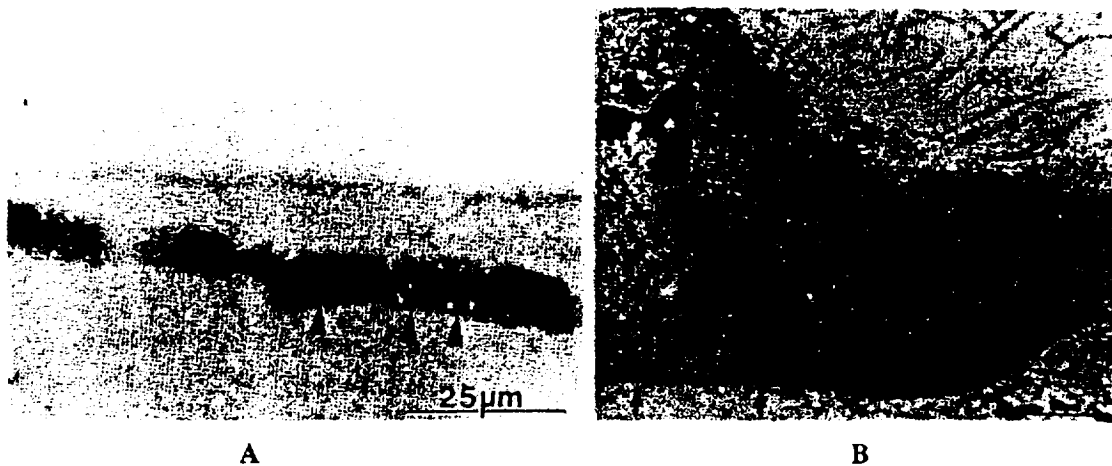


Fig. 2. In (A) an N-CAM immunoreactive regenerating myofiber is shown. (B) High magnification of a regenerating myofiber reveals that the cytoplasm contains abundant ribosomal aggregates associated with bundles of still disorganized myofilaments (MF). Immature Z bands (Z) are also conspicuous. A visible basement membrane (arrows) surrounds the cell. From D'Amelio and Danton (1992), with permission from the publisher.

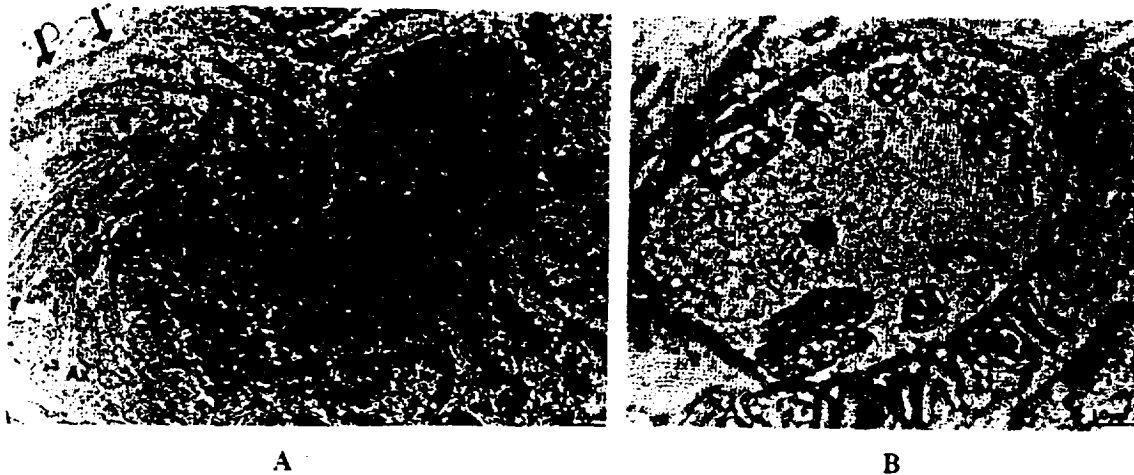


Fig. 3. (A) Synchronous control. Neuromuscular junction showing a preterminal axon (arrows) that gives rise to three axon terminals (Ax) apposing normal junctional folds. (B) Flight animal. The figure shows an axon profile almost devoid of synaptic vesicles and containing microtubular structures and few neurofilaments. From D'Amelio and Danton (1992), with permission from the publisher.



Fig. 4. (A) Flight animal. Neuromuscular junction displaying shrunken axon profiles (Ax1 and Ax2) occupied by myelin figures. Ax3 is completely devoid of synaptic vesicles. Schwann cell processes with degenerative alterations surround Ax1 and Ax2 (arrows) while Ax3 is covered by identifiable Schwann cell processes (arrowhead). (B) Flight animal. A myofiber undergoing necrosis (NF) shows dissolution of myofibrillar architecture, remains of altered myofibrils (*) and chromatin clumping and lysis of nuclei. A neuromuscular junction displays an elliptical axon profile (Ax) and junctional folds of apparently normal morphological characteristics. The reaction product of the synaptic cleft and junctional folds corresponds to esterase activity revealed by the staining procedure used to localize motor endplates. A small axon suggestive of an axonal sprout (arrow and inset) occupying the same post-synaptic space as the main axon terminal is separated from the latter by Schwann cell processes that also cover the sprout (arrowheads in inset). From D'Amelio and Danton (1992), with permission from the publisher.

Cerebral Cortex Study

The number of GABA-IR cells/mm² of the hindlimb representation was determined for each section lying within the boundary defined by the presence of the rostral hippocampus (Paxinos and Watson, 1986). A total of more than 7600 GABA-IR cells were identified. Cell counts on sections of HLS and control rats that were processed in the same immunostaining solutions were expressed for HLS as a percentage of control

$$\frac{(\text{HLS GABA-IR cells/mm}^2)}{(\text{CONTROL GABA-IR cells/mm}^2)} \times 100$$

GABA-IR cells were scattered in all cortical layers, but with the highest concentration in layer IV and lower concentrations in layers I and VI. The number of GABA-IR cells was reduced in rats subjected to HLS. Effects of HLS, expressed as the percentage of reduction in GABA-IR cells, in each cortical layer showed that the reduction in GABA-IR cells varied among cortical layers with significant reductions occurring in layers Va and Vb (32.75% and 22.07% respectively). Although quantitative assessment of GABAergic terminals ("puncta") targeting pyramidal cell soma and processes was not performed, it was obvious that they were markedly reduced in number in layers Va and Vb when compared with controls (Figure 5).

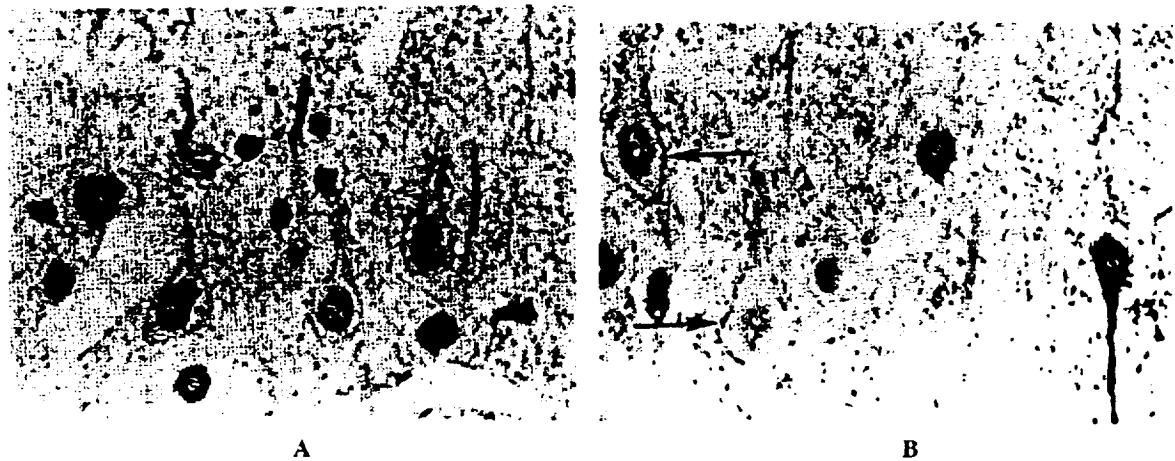


Fig. 5. Microphotographs of hindlimb somatosensory cortex at the level of layer Vb stained with GABA antiserum. (A) Tail-suspended animal. The pyramidal cells appear almost totally deprived of peripheral GABA-IR terminals. Note that the neuropil also shows very few terminals (arrowhead) as compared with the control in (B). (B) Control animal. Pyramidal cells surrounded by GABA-containing terminals (arrows). Numerous GABA-IR terminals are also conspicuous in the neuropil (arrowheads). PC, pyramidal cell; G, GABAergic cell. Magnification: 800x. From D'Amelio et al. (1996), with permission from the publisher.

DISCUSSION

A prolific literature exists on the numerous factors involved in triggering the process of muscle atrophy and subsequent deterioration of the myofibrillar structure in conditions of microgravity. The structural and metabolic foundations underlying these changes have been reviewed by Ilyin and Oganov (1989). Denervation-induced changes at the neuromuscular junctions have been reported in both spaceflight and ground based experiments (hindlimb unloading by tail suspension) as well (Riley *et al.*, 1990; Il'ina-

Kakueva and Portugalov, 1977; Baranski *et al.*, 1979; D'Amelio *et al.*, 1987; Pozdnyakov *et al.*, 1988). It is interesting to note that most of the alterations that we have described in the adductor longus muscle must have taken place during spaceflight and not as a consequence of post-flight exercise since the flight animals were sacrificed within approximately 3-11 hours after landing. It has been shown that it takes 2-3 days for typical mononucleated myoblasts to appear after muscle injury (Snow, 1977; Nichols and Shafiq, 1979). We believe that the extensive necrosis, with the possible overlapping effects of the denervation-reinnervation process, are the triggering factors for myofiber regeneration. In addition, the presence of innervation on regenerating myofibers suggests a process of remodeling of axon terminals. Axonal regeneration expressed by the visualization of small axon terminals (sprouting) was also seen on some necrotic fibers.

The presence of microtubules and neurofilaments found in some axon terminals almost totally devoid of synaptic vesicles is also intriguing. It seems reasonable to speculate that such appearance might be another indication of axonal remodeling. Such remodeling may be related to variations in the metabolism of motoneurons that trigger a reversal from a "transmitting" (stable) to a "growing" (plastic) state (Watson, 1976; Gordon, 1983). It has been shown that microtubules predominate during development and that during the regenerative response of motoneurons there is an increase in the ratio of tubulin to neurofilament which expresses a recapitulation of the more plastic states that take place during development (Hoffman and Lasek, 1980; Lasek, 1981).

The alterations of the neuromuscular junctions described in this report seem to suggest a process of denervation and remodeling during spaceflight, that is to say, a process limited to the "efferent" component of muscle innervation. Pronounced myofiber atrophy of antigravity muscles accompanied by severe alterations in a significant number of motor units have also been previously reported in HLS (D'Amelio *et al.*, 1987; see Edgerton and Roy for review, 1994). These findings, however, only represent a fragmentary view of the response of the neuromuscular system to spaceflight or HLS. Thus, we believed that further research in this area would profit from the development of a more "systemic" approach that would address questions such as, for example; what are the "functional" and/or morphological alterations of the "afferent" component of muscle due to the extensive lesions of the myofibers, and what are the effects on areas of the cerebral cortex related to inputs from muscle receptors. A natural result of this "systemic" approach would be a more thorough understanding of the adaptive capabilities of the organism to altered gravitational conditions. We thought then appropriate, as a following step, to initiate correlative studies on the most plastic structure of the central nervous system, the cerebral cortex, in animals subjected to "simulated" microgravity (HLS).

Consequences of limb unloading at the level of the cerebral cortex after spaceflight or HLS have not previously been addressed. Several lines of evidence lead us to suggest that the cortical changes reported here—reduction of GABA-IR cells and terminals in layer Va and Vb of the rat hindlimb somatosensory cortex—result from altered proprioceptive inputs from hindlimb muscle receptors with the possible participation of joint receptors and tendon organs. First, despite the changes described by us and others in muscle fibers and neuromuscular junctions, no morphological changes in muscle spindles or other sensory structures have been revealed by either light or electron microscopic observations. It is therefore likely that after HLS or SF sensory receptors continue to convey signals to the cerebral cortex from "slow" weight bearing muscles (e.g. soleus, adductor longus), as well as from the predominantly "fast" non-weight bearing muscles (e.g., tibialis anterior) of the hind limbs.

Second, since receptors of the affected "slow" extensors (e.g., soleus) and the relatively unaffected "fast" extensors (e.g., lateral and medial gastrocnemius) and "fast" flexors (e.g., tibialis anterior) apparently remain operative following HLS or SF, a mismatch of afferent messages from these muscles to the cerebral cortex should be expected. Since in normal conditions stretching of the antigravity soleus muscle evokes heterogenic reflexes in lateral and medial gastrocnemius and tibialis anterior (Nichols, 1989; see also Cope *et al.*, 1994), an imbalance in the reflex responses of these synergistic muscles is most likely responsible for the disruption of gait previously demonstrated by us following HLS and SF (Fox *et al.*, 1993, 1994). That such an imbalance can lead to changes in the cerebral cortex has been demonstrated by Sanes *et al.* (1992). These investigators have suggested that sensory inputs from muscle receptors are used to adjust the neural circuits related to the specific output functions of the motor cortex and that a mismatch between cortical outputs and sensory inputs during active limb movements (e.g., during walking) can lead to the

reorganization of the cortical motor outputs. Such goal-directed reorganization would be designed to optimize function (e.g., walking) under the conditions of altered inputs from hindlimb muscles.

Thus, the modification of sensory inputs to the central nervous system due to altered functioning of hindlimb muscles, along with the requirements for reprogramming of motor outputs to compensate for the changes in structure and function of those same muscles, could lead to plastic modifications of the circuitry at the cortical level. In these modifications local circuit GABAergic neurons of the cerebral cortex are the most logical candidates to modulate the discharge frequency of pyramidal cells (see Jones, 1993) since: a) The majority of identified local circuit neurons in the cerebral cortex are GABAergic (White, 1989), b) GABAergic cells are present in all layers of the mammalian cerebral cortex (Ribak, 1978; Houser *et al.*, 1984; White, 1989) and c) The main synaptic target of all classes of GABAergic neurons are the pyramidal cells and their processes (White, 1989). Furthermore, experiments primarily concerned with neuronal receptive fields in the somatosensory cortex have shown that GABA-mediated intracortical inhibition specifies size and thresholds of receptive fields of major neuronal subgroups (Hicks and Dykes, 1983; Dykes *et al.*, 1984; see also Jacobs and Donoghue, 1991). It has been shown that the cortical substrate subserving tactile and proprioceptive limb placing—that is deeply disturbed after HLS (Fox, unpublished data)—coincide with a dense subfield of large pyramidal neurons in the deeper part of layer V (De Ryck *et al.*, 1992). In our experiments, layer V showed the most pronounced reduction of GABA-IR cells.

In short, as a result of the selective and differential effects of HLS on weight and non-weight bearing muscles, corticospinal fibers would influence motoneuronal pools with either a significant number of abnormal axon terminals innervating the atrophic antigravity muscles or with normal axon terminals innervating non-weight bearing muscles having minimal or no alterations. As a consequence, disturbances in the afferent signaling and feedback information from intramuscular receptors (particularly muscle spindles) to the cerebral cortex would trigger an imbalance in the reflex organization of these synergistic muscle groups. In turn, pyramidal tract neurons processing altered sensory information would respond with changes in the rates of discharge that are modulated by GABAergic neurons.

The emphasis put on muscle spindles over other receptor types as responsible for the changes has a reason, although admittedly speculative. Electrophysiological studies of the rat somatosensory cortex suggest an overlap (co-extension) of sensory and motor areas ("sensorimotor amalgam"), *particularly at the level of the hindlimb representation* where layer V contains large pyramidal cells that extend over, without interruption, from the motor cortex (Hall and Lindholm, 1974). This type of cortical organization would seem to lend support to the hypothesis first proposed by Phillips (1969) that information from muscle spindles to the cerebral cortex is relayed through an oligosynaptic transcortical spindle circuit for proprioceptive signals whose efferent limb is the corticomotoneuronal projection (see Hummelsheim and Wiesendanger, 1985, for discussion). Several subsequent studies have provided more evidence in favor of this hypothesis (see Landgren and Silfvenius, 1969, 1971; McIntyre, 1974; Wiesendanger and Miles, 1982; Matthews, 1991). Whether the decrease in GABA immunoreactivity is due to alterations in its synthetic activity or depletion due to increased release is a matter of speculation that will require additional studies (e.g., *in situ* hybridization). Furthermore, electrophysiological recordings will be necessary to assess patterns of activity and receptive field size of cortical neurons influenced by GABA-mediated inhibition under the same conditions. Since the changes we have described are presumably transient (normal gait is recovered after several weeks—Fox *et al.*, 1993, 1994) it would be important to investigate changes in GABA-IR during the recovery process, and to assess whether these alterations may become irreversible given a sufficiently long period of hindlimb unloading.

Changes in GABA-IR were previously reported under conditions of sensory deprivation by surgical means. For example, Warren *et al.* (1989) reported a 16% decrease of glutamic acid decarboxylase (GAD) immunoreactive cells in layer IV of the rat hindlimb somatosensory cortex 2 weeks after transection of the sciatic nerve. In experiments conducted in monkey visual cortex after 2-3 weeks of eye enucleation, Hendry and Jones (1986) found a 45% reduction of GABA-IR cells in layer IV. In the same region, these investigators also showed a 36% decrease of GABA-IR cells 11 weeks after eyelid suture.

Unlike surgical deafferentation, in HLS the afferent input is not interrupted but rather significantly disrupted by non-invasive unloading of weight-bearing muscles. Our results suggest that non-invasive manipulations of the neuromuscular system, e.g., HLS or SF, can have significant effects on cortical circuitry. Other lines of work based on non-invasive procedures support this possibility (see for example, Jenkins *et al.*, 1990;

Merzenich *et al.*, 1990; Sanes *et al.*, 1992). Since the central nervous system must constantly adjust movements in response to altered environmental conditions, we believe that studies in intact animals should be pursued to help clarify the mechanisms of cortical plasticity and adaptation under natural conditions.

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REFERENCES

- Akhtar, N. D., and P. W. Land, Activity-Dependent Regulation of Glutamic Acid Decarboxylase in the Rat Barrel Cortex: Effects of Neonatal Versus Adult Sensory Deprivation, *J. Comp. Neurol.*, **307**, 200 (1991).
- Baranski, S., W. Baranska, M. Marciniak, and E. I. Ilyina-Kakueva, Ultrasonic ("Ultrastructural") Investigations of the Soleus Muscle after Space Flight on the Biosputnik 936, *Aviat. Space Environ. Med.*, **50**, 930 (1979).
- Cope, T. C., S. J., Bonasera, and T. R. Nichols, Reinnervated Muscles Fail to Produce Stretch Reflexes, *J. Neurophysiol.*, **71**, 817 (1994).
- D'Amelio, F., and N. G. Daunton, Effects of Spaceflight in the Adductor Longus Muscle of Rats Flown in the Soviet Biosatellite Cosmos 2044: A Study Employing Neural Cell Adhesion Molecule (N-Cam) Immunocytochemistry and Conventional Morphological Techniques (Light and Electron Microscopy), *J. Neuropath. Exp. Neurol.*, **51**, 415 (1992).
- D'Amelio, F., N. G. Daunton, T. Fast, and R. Grindeland, Preliminary Findings in the Neuromuscular Junctions of the Soleus Muscle of Adult Rats Subjected to Simulated Weightlessness. Light and Electron Microscopy, *Space Life Sciences Symposium: Three Decades Of Life Science Research In Space*, Washington, D.C., pp. 204-205 (1987).
- D'Amelio, F., R. A. Fox, L. C. Wu, and N. G. Daunton, Quantitative Changes of GABA-Immunoreactive Cells in the Hindlimb Representation of the Rat Somatosensory Cortex after 14-Day Hindlimb Unloading by Tail Suspension, *J. Neurosci. Res.*, **44**, 532 (1996).
- De Ryck, M., J. Van Reempts, H. Duytschaever, B. Van Deuren, and G. Clincke, Neocortical Localization of Tactile/Proprioceptive Limb Placing Reactions in the Rat, *Brain Res.*, **573**, 44 (1992).
- Dykes, R. W., P. Landry, R. Metharate, and T. P. Hicks, Functional Role of GABA in Cat Primary Somatosensory Cortex: Shaping Receptive Fields of Cortical Neurons, *J. Neurophysiol.*, **52**, 1066 (1984).
- Edgerton, V. R., and R. R. Roy, Neuromuscular Adaptation to Actual and Simulated Spaceflight, in *Handbook of Physiology, Section 4, Vol. 1, Adaptation to The Environment*, edited by M. J. Fregly, and C. M. Blatteis, pp. 721-763, Oxford University Press, New York (1994).
- Fox, R. A., M. Corcoran, N. G. Daunton, and E. Morey-Holton, Effects of Spaceflight and Hindlimb Suspension on the Posture and Gait of Rats, in *Vestibular And Neural Front*, edited by K. Taguchi, M. Igarashi, and S. Mori, pp. 603-606, Elsevier, Amsterdam (1994).

- Fox, R. A., N. G. Daunton, M. L. Corcoran, L. C. Wu, and F. D'Amelio, Tail Suspension With and Without Hindlimb Unloading Affects Neuromuscular Function in the Adult Rat, *Neuroscience Abstracts*, **19**(1), 147 (1993).
- Gordon, T., Dependence of Peripheral Nerves on Their Target Organs, in *Somatic And Autonomic Nerve-Muscle Interaction*, edited by G. Burnstock, R. O'Brien, and G. Vrbová, pp. 289-325, Elsevier, Amsterdam (1983).
- Hall, R. D., and E. P. Lindholm, Organization of Motor and Somatosensory Neocortex in the Albino Rat, *Brain Res.*, **66**, 23 (1974).
- Hendry, S. H. C., and E. G. Jones, Reduction in Number of Immunostained GABAergic Neurones in Deprived-Eye Dominance Columns of Monkey Area 17, *Nature*, **320**, 750 (1986).
- Hicks, T. P., and R. W. Dykes, Receptive Field Size for Certain Neurons in Primary Somatosensory Cortex is Determined by GABA-Mediated Intracortical Inhibition, *Brain Res.*, **274**, 160 (1983).
- Hoffman, P. N., and R. J. Lasek, Axonal Transport of the Cytoskeleton in Regenerating Motor Neurons: Constancy and Change, *Brain Res.*, **202**, 317 (1980).
- Houser, C. R., J. E. Vaughn, S. H. C. Hendry, E. G. Jones, and A. Peters, GABA Neurons in the Cerebral Cortex, in *Cerebral Cortex. Functional Properties of Cortical Cells*, Vol.2, edited by E. G. Jones, and A. Peters, pp. 63-89, Plenum Press, New York (1984).
- Hummelsheim, H., and M. Wiesendanger, Is the Hindlimb Representation of the Rat's Cortex a 'Sensorimotor Amalgam'?, *Brain Res.*, **346**, 75 (1985).
- Il'ina-Kakueva, Ye. I., and V. V. Portugalov, State of Rat Muscle Motoneuron System in the Case of Restricted Mobility, *Kosmicheskaya Biologiya I Aviakomicheskaya Meditsina*, **6**, 31 (1977).
- Ilyin, E. A., and V. S. Oganov, Microgravity and Musculoskeletal System of Mammals, *Adv. Space Res.*, **9**, 11 (1989).
- Jacobs, K. M., and J. P. Donoghue, Reshaping the Cortical Motor Map by Unmasking Latent Intracortical Connections, *Science*, **251**, 944 (1991).
- Jenkins, W. M., M. M. Merzenich, M. T. Ochs, T. T. Allard, and E. Guic-Robles, Functional Reorganization of Primary Somatosensory Cortex in Adult Owl Monkeys after Behaviorally Controlled Tactile Stimulation, *J. Neurophysiol.*, **63**, 82 (1990).
- Jones, E. G., The Role of Afferent Activity in the Maintenance of Primate Neocortical Function. *J. Exp. Biol.*, **153**, 155 (1990).
- Jones, E. G., GABAergic Neurons and Their Role in Cortical Plasticity in Primates, *Cerebral Cortex*, **3**, 361 (1993).
- Landgren, S., and H. Silfvenius, Projection to Cerebral Cortex of Group I Muscle Afferents from the Cat's Hind Limb, *J. Physiol.*, **200**, 353 (1969).
- Landgren, S., and H. Silfvenius, Nucleus Z, the Medullary Relay in the Projection Path to the Cerebral Cortex of Group I Muscle Afferents from the Cat's Hindlimb, *J. Physiol.*, **218**, 551 (1971).
- Lasek, R. J., The Dynamic Ordering of Neuronal Cytoskeletons, *Neurosci. Res. Prog. Bull.*, **19**, 7 (1981).
- Matthews, P. B., The Human Stretch Reflex and the Motor Cortex, *Trends in Neurosci.*, **14**, 87 (1991).

- McIntyre, A. K., Central Actions of Impulses in Muscle Afferent Fibers, in *Muscle Receptors*, edited by D. Barker, C. C. Hunt, and A. K. McIntyre, pp. 235-288, Springer, New York (1974).
- Merzenich, M. M., G. H. Recanzone, W. M. Jenkins, and R. J. Nudo, How the Brain Functionally Rewires Itself, in *Natural and Artificial Parallel Computation*, edited by M. A. Arbib, and J. A. Robinson, pp.177-210, The MIT Press, Cambridge, Massachusetts (1990).
- Nichols, T. R., The Organization of Heterogenic Reflexes Among Muscles Crossing the Ankle Joint in the Decerebrate Cat, *J. Physiol. (Lond.)*, **410**, 463 (1989).
- Nichols, J. R., and S. A. Shafiq, Muscular Regeneration in the Muscular Dystrophies. *Ann. N. Y. Acad. Sci.*, **317**, 478 (1979).
- Paxinos, G., and C. Watson, *The Rat Brain in Stereotaxic Coordinates*, Academic Press, New York (1986).
- Phillips, C. G., The Ferrier Lecture 1968. Motor Apparatus of the Baboon's Hand. *Proc. R. Soc. London Ser.B*, **173**, 141 (1969).
- Pozdnyakov, O. M., L. L. Babakova, M. S. Demorzhii, and E. I. Ilyina-Kakueva, Changes in Rat Neuro-Muscular Synapse Ultrastructure During Space Flights, *Bull. Exp. Biol. Med.*, **6**, 752 (1988).
- Ribak, C. E., Aspinous and Sparsely-Spinous Stellate Neurons in the Visual Cortex of Rats Contain Glutamic Acid Decarboxylase, *J. Neurocytol.*, **7**, 461 (1978).
- Riley, D. A., E. I. Ilyina-Kakueva, S. Ellis, J. L. W. Bain, G. R. Slocum, and F. R. Sedlak, Skeletal Muscle Fiber, Nerve, and Blood Vessel Breakdown in Space-Flown Rats, *FASEB Journal*, **4**, 84 (1990).
- Sanes, J. N., J. Wang, and J. P. Donoghue, Immediate and Delayed Changes of Rat Motor Cortical Output Representation with New Forelimb Configurations, *Cerebral Cortex*, **2**, 141 (1992).
- Snow, M. H., Myogenic Cell Formation in Regenerating Rat Skeletal Muscle Injured by Mincing. I. A Fine Structural Study, *Anat. Rec.*, **188**, 181 (1977).
- Thomason, D. B., and F. W. Booth, Atrophy of the Soleus Muscle by Hindlimb Unweighting, *J. Appl. Physiol.*, **68**, 1 (1990).
- Warren, R., N. Tremblay, and R. W. Dykes, Quantitative Study of Glutamic Acid Decarboxylase-Immunoreactive Neurons and Cytochrome Oxidase Activity in Normal and Partially Deafferented Rat Hindlimb Somatosensory Cortex, *J. Comp. Neurol.*, **288**, 583 (1989).
- Watson, W. E, *Cell Biology of Brain*, John Wiley, New York (1976).
- White, E., *Cortical Circuits. Synaptic Organization of the Cerebral Cortex. Structure, Function and Theory*. Birkhäuser, Boston (1989).
- Wiesendanger, M., and T. S. Miles, Ascending Pathways of Low Threshold Muscle Afferents to the Cerebral Cortex and Its Possible Role in Motor Control, *Physiol. Rev.*, **62**, 1234 (1982).

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QUANTITATIVE CHANGES OF GABA-IMMUNOREACTIVE CELLS IN THE HINDLIMB REPRESENTATION OF THE RAT SOMATOSENSORY CORTEX AFTER 14-DAY HINDLIMB UNLOADING BY TAIL SUSPENSION. E.

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In the present study γ -aminobutyric acid (GABA) immunoreactivity was evaluated quantitatively in the hindlimb representation of the rat somatosensory cortex after 14 days of tail suspension (TS). The number of GABA-immunoreactive cells was reduced in cortical layers IV, Va, Vb and VI ($p < 0.05$, $p < 0.0002$, $p < 0.02$, and $p < 0.03$ respectively) of rats subjected to TS. In addition, the number of GABA-containing terminals, particularly those terminals surrounding the soma and apical dendrites of pyramidal cells in layer Vb, also were reduced throughout the same cortical layers. Since there was no reduction in the total neuronal density of the hindlimb representation as compared with the control animals, we concluded that the reduction was not due to cell death. Findings are discussed in the context of previous morphological and behavioral studies of the neuromuscular system of TS animals and we propose that alterations in the reflex organization of hindlimb muscle groups that are triggered by TS elicit disturbances in the afferent signaling and feedback from intramuscular receptors to the cerebral cortex. We suggest that local circuit GABAergic neurons modulate cortical output in response to this altered afferent feedback.

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Quantitative Changes of GABA-Immunoreactive Cells in the Hindlimb Representation of the Rat Somatosensory Cortex After 14-Day Hindlimb Unloading by Tail Suspension

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The present study was aimed at evaluating quantitatively γ -aminobutyric acid (GABA) immunoreactivity in the hindlimb representation of the rat somatosensory cortex after 14 days of hindlimb unloading by tail suspension. A reduction in the number of GABA-immunoreactive cells with respect to the control animals was observed in layer Va and Vb. GABA-containing terminals were also reduced in the same layers, particularly those terminals surrounding the soma and apical dendrites of pyramidal cells in layer Vb. On the basis of previous morphological and behavioral studies of the neuromuscular system of hindlimb-suspended animals, it is suggested that the unloading due to hindlimb suspension alters afferent signaling and feedback information from intramuscular receptors to the cerebral cortex due to modifications in the reflex organization of hindlimb muscle groups. We propose that the reduction in immunoreactivity of local circuit GABAergic neurons and terminals is an expression of changes in their modulatory activity to compensate for the alterations in the afferent information. © 1996 Wiley-Liss, Inc.

Key words: immunocytochemistry, cerebral cortex, muscle, NIH-Image

INTRODUCTION

Hindlimb unloading by tail suspension (HLS) is a non-invasive procedure that simulates some of the effects of weightlessness on antigravity muscles (e.g., soleus atrophy) observed following spaceflight (SF) (Ilyin and Oganov, 1989; Thomason and Booth, 1990; Edgerton and Roy, 1994, for reviews). Although the consequences of unloading have been well-studied in the muscles, little attention has been paid to the possible effects of hindlimb unloading on those areas of the central nervous system related to sensory inputs from muscles.

The primary concern of this experiment was to determine whether γ -aminobutyric acid (GABA) immunoreactivity (GABA-IR) of local circuit cortical neurons could be altered as a result of a non-invasive procedure such as HLS. Our assumption—based on our current behavioral and morphological studies (D'Amelio et al., 1987; D'Amelio and Daunton, 1992; Fox et al., 1993, 1994)—was that muscle atrophy produced by HLS could modify sensory inputs arising from muscle receptors to the cerebral cortex.

We have focused the present report on the behavior of GABAergic neurons since numerous lines of research have demonstrated modifications in the level of GABA-IR or glutamic acid decarboxylase (GAD) immunoreactivity in cortical interneurons when sensory activity is altered by surgical manipulation (Hendry and Jones, 1986; Warren et al., 1989; Akhtar and Land, 1991; see also Jones, 1990).

MATERIALS AND METHODS

Animals

Six Sprague-Dawley rats (200–250 g) were employed for this study. Three served as controls and three were suspended (HLS) by the tail for 14 days.

Suspension Procedure

The suspension procedure (Wronski and Holton, 1987) consisted of the following steps: the tail was cleaned with gauze previously soaked in 70% ethanol, then sprayed with tincture of benzoin for protection

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against adhesive tape irritation, and allowed to dry. A strip of orthopedic tape was attached to a plastic suspension bar and applied to the lateral sides of the tail. The tape was then secured by wrapping a strip of stockette around the tail. The plastic suspension bar was then attached to a pulley system mounted on the top of an acrylic housing unit. In this manner the unloading of the hindlimbs was achieved while the forelimbs were used for locomotion and unimpeded access to food and water. Body weight was recorded daily. Control rats were housed individually in similar cages located in the same room but had no attachments to the tail. The room was maintained at 24°C with a 12-hr light/dark cycle.

Fixation and Sectioning

After 14 days of tail suspension the animals and their controls were deeply anesthetized with Metophane and immediately perfused via the heart with 50 ml 0.9% saline, followed by 500 ml of a fixative made up of 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed the same day, immersed in fresh fixative, and stored at 4°C.

The right hemisphere was coronally blocked between Bregma -1.8 mm and Bregma -3.6 mm, where the somatosensory representation of the hindlimb is conspicuous and associated with the presence of the rostral hippocampus (Paxinos and Watson, 1986). At this level the more rostrally located forelimb representation is no longer present (rostral to Bregma -1.8 the somatosensory cortex contains both hindlimb and the laterally adjacent forelimb representations. The hippocampus is not visible). Coronal sections 40 μ m thick were cut on a Vibratome and collected in TBS (0.05 M Tris buffer-0.9% saline, pH 7.6). Twenty serial sections per animal were used for the staining procedures; 15 were stained for immunocytochemistry, and 5 were Nissl stained with cresyl violet to identify the cytoarchitectonic layers of the hindlimb representation.

GABA Immunocytochemistry

Floating sections were first incubated for 5–10 min at room temperature (RT) with 3% hydrogen peroxide in 10% methanol in TBS and subsequently rinsed 4 times in TBS \times 30 min (RT). The sections were then immersed in GABA antiserum (Chemicon, cat. no. AB131) or control serum (preimmune rabbit serum) diluted at 1:1,000 in TBS for 48–72 hr at 4°C, with orbital agitation. Then they were rinsed 4 times in TBS \times 30 min (RT) and incubated for 60 min (RT) in swine anti-rabbit IgG diluted 1:50 in TBS. The sections were rinsed 4 more times in TBS \times 30 min (RT) and then incubated for 60 min (RT) with rabbit peroxidase-antiperoxidase complex (Sigma) diluted 1:200 in TBS. To develop reaction product the sections were immersed in 12.5 mg diaminoben-

zidine tetrahydrochloride in 50 ml TBS + 5 μ l 30% hydrogen peroxide for 5–8 min. Finally, they were rinsed in TBS, 2 changes \times 10 min (RT), mounted on gelatin coated slides, air-dried, and coverslipped with Permount.

The sections from pairs of experimental and control animals were processed together in the same solutions for consistent immunostaining. For identification purposes, the hemisphere of the control rat was marked with a small hole at the level of the striatum. Sections of each suspended and control pair were placed on the same glass slide for counting of GABA-IR cells.

Methodology for Quantitative Analysis

A Bausch & Lomb inverted microscope equipped with a 25 \times objective was employed to complete the first steps of the analysis. The microscope was set on a table to project the image of the slides at 58 \times magnification.

The hindlimb somatosensory cortex was identified in Nissl-stained slides by the prominent aggregation of granular cells in layer IV. The boundaries of the hindlimb representation were drawn on a piece of white paper. The projected image of the sections stained with GABA antiserum was superimposed on the drawing, and GABA-IR cells intensely or moderately stained were marked on the paper. Blood vessels as well as meningeal foldings served as reference marks for each section. The marking of the cells slightly exceeded the lateral and medial boundaries of the hindlimb representation. Subsequently, the coverslips of the anti-GABA stained slides were removed by soaking in xylene, and the sections were Nissl-stained with cresyl violet and remounted. The Nissl staining of the slides in which the marking of the GABA-IR cells was previously made gave us more confidence in tracing the boundaries of the area and demarcating the cortical laminae based on the prominent granular aggregates of layer IV. The projected image of these sections was drawn on a translucent sheet of paper. The drawing included the boundaries of the hindlimb representation, the reference marks, and the dividing lines of six cortical layers identified as layers I, II/III, IV, Va, Vb, and VI (see Zilles and Wree, 1985). This drawing was then overlaid on the paper that had the markings of GABA-IR cells. The boundaries of the hindlimb cortex were then corrected and GABA-IR cells were counted in each layer on the translucent paper (Figs. 1, 2).

The image of each layer on this translucent sheet was captured into a Macintosh Centris 650 computer using a Sierra Scientific Model MS4030 CCD tube camera that had a macro Nikon/Nikor 55 lens and a Scion Technology LG-3 frame grabber board in the Nubus slot of the computer. Version 1.54 of the public domain NIH-Image image analysis software (written by Wayne Rasband at the U.S. National Institutes of Health) was used

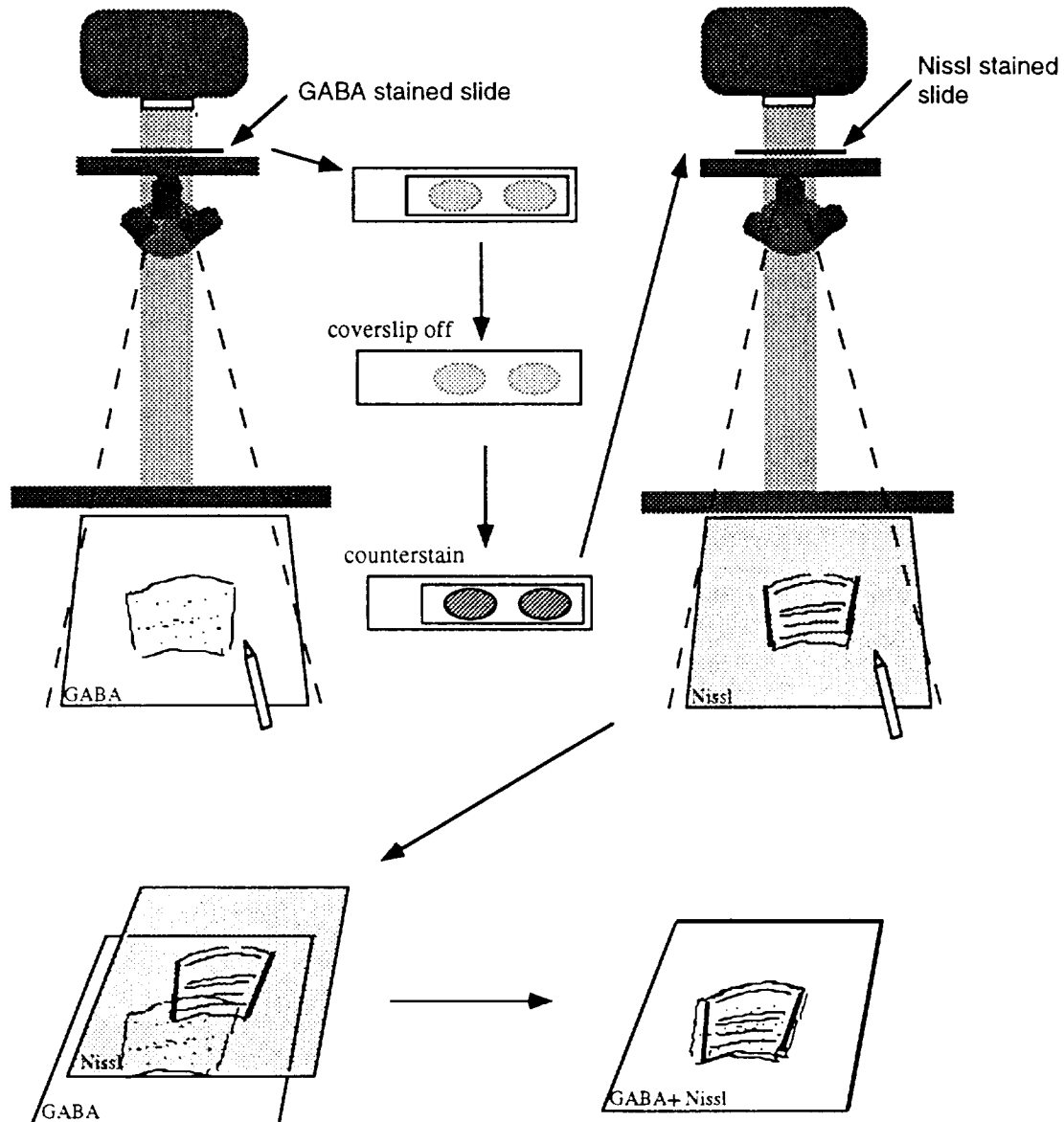


Fig. 1. Schematic drawing of method used for quantitative analysis. See text for details.

for image acquisition and for area measurement of each of the six layers. (The software is available electronically via Internet by anonymous ftp from zippy.nimh.nih.gov or from Library 9, the MacApp forum on CompuServe and on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22161, Part number PB93-504868.)

An image of standard square inches etched in the copy stand was also captured and then used to compute the correction factor for the distortion of the aspect ratio introduced by the camera lens and the computer monitor. Quantitative measurements of the cortical layers were done blind by one of us (L.C.W.). The digitized images were magnified at $2\times$, and a sharpening filter was used

prior to measuring. Measurements are based on four to eight GABA/Nissl-stained slides for each of the three rats in each group. The measurement data and the number of GABA-IR cells for each layer were entered into Microsoft Excel v.4.0. The frequency of GABA-IR cells/mm² area in each layer for each treatment group was then computed.

RESULTS

The number of GABA-IR cells/mm² of the hind-limb representation was determined for each section lying within the boundary defined by the presence of the

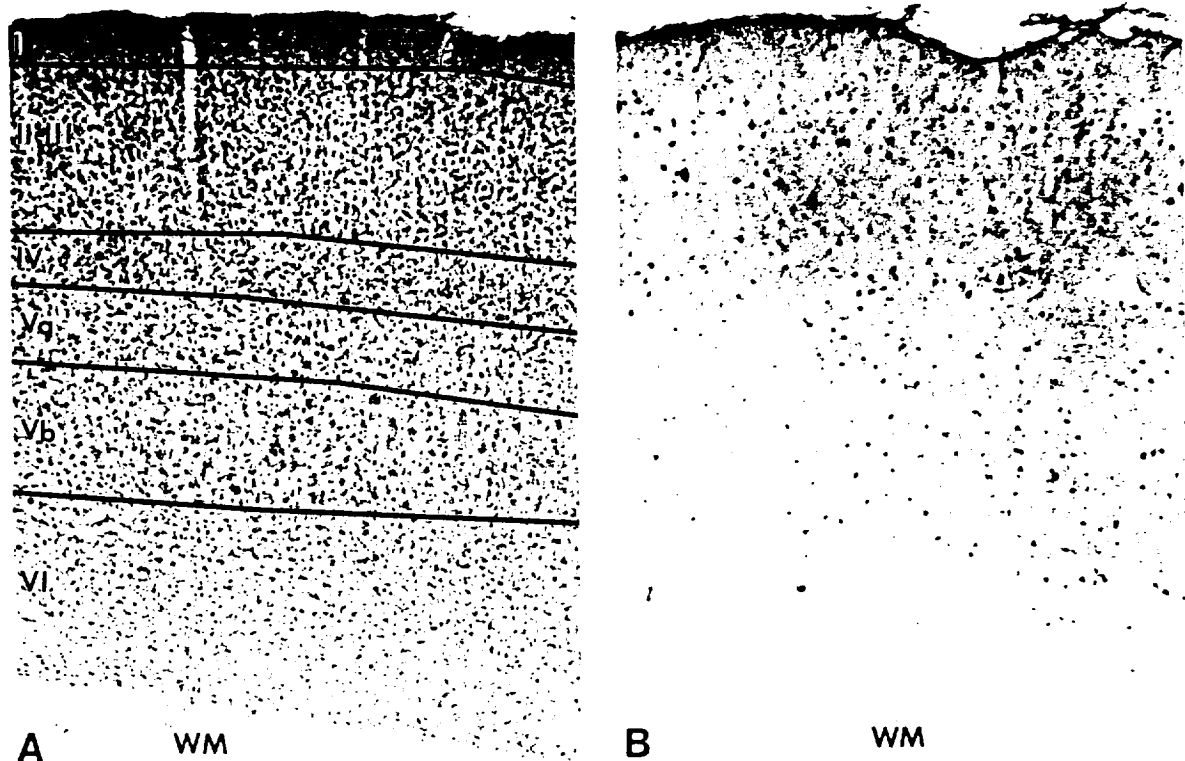


Fig. 2. A: Microphotograph of a Nissl-stained coronal section through the hindlimb somatosensory cortex showing the demarcation of the six layers in which counting of GABA-IR cells was done. Note the aggregation of granular cells in layer IV. B: Same level as in A, stained with GABA antiserum. WM, white matter. Magnification $\times 55$.

rostral hippocampus. A total of more than 7,600 GABA-IR cells were identified. Cell counts were based on four sections for one rat subjected to HLS, on six sections for one control rat, and on eight sections for the four (two HLS and two control) remaining rats. To eliminate differences in staining between pairs of rats, cell counts on sections of HLS and control rats that were processed in the same immunostaining solutions were expressed for HLS as a percentage of control

$$\frac{(\text{HSL GABA-IR cells/mm}^2)}{(\text{CONTROL GABA-IR cells/mm}^2)} \times 100.$$

GABA-IR cells were scattered in all cortical layers, but with the highest concentration in layer IV and lower concentrations in layers I and VI. The number of GABA-IR cells was reduced in rats subjected to HLS. Effects of HLS, expressed as the percentage of reduction in GABA-IR cells, in each cortical layer is shown in Table I. As seen in this table, the reduction in GABA-IR cells varied among cortical layers, with significant reductions occurring in layers Va and Vb.

Although quantitative assessment of GABAergic

terminals ("puncta") targeting pyramidal cell soma and processes was not performed, it was obvious that they were markedly reduced in number in layers Va and Vb when compared with controls (Fig. 3).

DISCUSSION

While it is well documented that the unloading of antigravity muscles by hindlimb suspension leads to atrophy, alterations of neuromuscular units, changes in contractile properties, and the loss of coordination of muscular contraction among different muscle groups (for review, see Edgerton and Roy, 1994), consequences of the unloading at the level of the cerebral cortex have not previously been addressed. Our results indicate that unloading of the hindlimbs results in a significant reduction in immunoreactivity of GABAergic cells and terminals in layers Va and Vb of the rat hindlimb somatosensory cortex.

Several lines of evidence lead us to suggest that the cortical changes reported in this study result from altered proprioceptive inputs from hindlimb muscle receptors without neglecting the possibility of participation of joint

TABLE I. Percentage of GABA-IR Cells in HLS Relative to Control Rats

	Cortical layer					
	I	II/III	IV	Va	Vb	VI
Pair A HLS/C %	108.03	93.56	116.38	65.01	87.35	113.13
Pair B HLS/C %	81.23	111.45	87.48	75.72	81.55	64.60
Pair C HLS/C %	95.72	62.61	57.84	61.02	64.90	47.26
Mean	94.99	89.21	87.23	67.25	77.93	75.00
SD	13.41	24.71	29.27	7.60	11.65	34.14
SEM	7.74	14.27	16.90	4.39	6.73	19.71
% Decrease	5.01	10.79	12.77	32.75	22.07	25.00
<i>t</i> test	0.65	0.76	0.76	7.46	3.28	1.27
<i>P</i> value	>0.10	>0.10	>0.10	<0.01*	<0.05*	>0.10

*Significant difference, by one-tailed test.

receptors and tendon organs. First, although we have previously shown pronounced myofiber atrophy of anti-gravity muscles accompanied by severe alterations in a significant number of motor units immediately after HLS or SF, i.e., degeneration of axon terminals and decrease in the number or absence of synaptic vesicles (D'Amelio et al., 1987; D'Amelio and Dauntun, 1992; for review, see Edgerton and Roy, 1994), no morphological changes in muscle spindles or other sensory structures have been revealed by either light or electron microscopic observations of the same material (D'Amelio, unpublished results). It is therefore likely that after HLS or SF sensory receptors continue to convey signals to the cerebral cortex from "slow" weight-bearing muscles (e.g., soleus), as well as from the predominantly "fast" non-weight-bearing muscles (e.g., tibialis anterior) of the hind limbs.

Second, since receptors of the affected "slow" extensors (e.g., soleus) and the relatively unaffected "fast" extensors (e.g., lateral and medial gastrocnemius) and "fast" flexors (e.g., tibialis anterior) apparently remain operative following HLS or SF, a mismatch of afferent messages from these muscles to the cerebral cortex should be expected. Since in normal conditions stretching of the antigravity soleus muscle evokes heterogenic reflexes in lateral and medial gastrocnemius and tibialis anterior (Nichols, 1989; see also Cope et al., 1994), an imbalance in the reflex responses of these synergetic muscles is most likely responsible for the disruption of gait previously demonstrated by us following HLS and SF (Fox et al., 1993, 1994). That such an imbalance can lead to changes in the cerebral cortex has been demonstrated by Sanes et al. (1992). These investigators have suggested that sensory inputs from muscle receptors are used to adjust the neural circuits related to the specific output functions of the motor cortex and that a mismatch between cortical outputs and sensory inputs during active limb movements (e.g., during walking) can lead to the reorganization of the cortical motor outputs. Such goal-directed reorganization would be designed to

optimize function (e.g., walking) under the conditions of altered inputs from hindlimb muscles.

Thus, the modification of sensory inputs to the central nervous system due to altered functioning of hindlimb muscles, along with the requirements for reprogramming of motor outputs to compensate for the changes in structure and function of those same muscles, could lead to plastic modifications of the circuitry at the cortical level. In these modifications local circuit GABAergic neurons of the cerebral cortex are the most logical candidates to modulate the discharge frequency of pyramidal cells (Jones, 1993) since: 1) the majority of identified local circuit neurons in the cerebral cortex are GABAergic (White, 1989); 2) GABAergic cells are present in all layers of the mammalian cerebral cortex (Ribak, 1978; Houser et al., 1984; White, 1989); and 3) the main synaptic targets of all classes of GABAergic neurons are the pyramidal cells and their processes (White, 1989). Furthermore, experiments primarily concerned with neuronal receptive fields in the somatosensory cortex have shown that GABA-mediated intracortical inhibition specifies size and thresholds of receptive fields of major neuronal subgroups (Hicks and Dykes, 1983; Dykes et al., 1984; see also Jacobs and Donoghue, 1991). It has been shown that the cortical substrate subserving tactile and proprioceptive limb placing—which is deeply disturbed after HLS (Fox, unpublished data)—coincides with a dense subfield of large pyramidal neurons in the deeper part of layer V (De Ryck et al., 1992). In our experiments, layer V showed the most pronounced reduction of GABA-IR cells.

In short, as a result of the selective and differential effects of HLS on weight- and non-weight-bearing muscles, corticospinal fibers would influence motoneuronal pools with either a significant number of abnormal axon terminals innervating the atrophic antigravity muscles or with normal axon terminals innervating non-weight-bearing muscles having minimal or no alterations. As a consequence, disturbances in the afferent signaling and



Fig. 3. Microphotographs of hindlimb somatosensory cortex at the level of layer Vb stained with GABA antiserum. **A:** Tail-suspended animal. The pyramidal cells appear almost totally deprived of peripheral GABA-IR terminals. Note that the neuropil also shows very few terminals (arrowhead) compared with the control in **B**. **B:** Control animal. Pyramidal cells are surrounded by GABA-containing terminals (arrow). Numerous GABA-IR terminals are also conspicuous in the neuropil (arrowheads). PC, pyramidal cell; G, GABAergic cell. Magnification $\times 800$.

feedback information from intramuscular receptors (particularly muscle spindles) to the cerebral cortex would trigger an imbalance in the reflex organization of these synergetic muscle groups. In turn, pyramidal tract neurons processing altered sensory information would respond with changes in the rates of discharge that are modulated by GABAergic neurons.

The emphasis put on muscle spindles over other receptor types as responsible for the changes has a reason, although admittedly speculative. Electrophysiological studies of the rat somatosensory cortex suggest an overlap (co-extension) of sensory and motor areas ("sensorimotor amalgam"), *particularly at the level of the hindlimb representation* where layer V contains large pyramidal cells that extend over, without interruption, from the motor cortex (Hall and Lindholm, 1974). This type of cortical organization would seem to lend support to the hypothesis first proposed by Phillips (1969) that information from muscle spindles to the cerebral cortex is relayed through an oligosynaptic transcortical spindle circuit for proprioceptive signals whose efferent limb is the corticomotoneuronal projection (for discussion, see Hummelsheim and Wiesendanger, 1985). Several subsequent studies have provided more evidence in favor of this hypothesis (Landgren and Silfvenius, 1969, 1971; McIntyre, 1974; Wiesendanger and Miles, 1982; Matthews, 1991).

Whether the decrease in GABA immunoreactivity is due to alterations in its synthetic activity or depletion due to increased release is a matter of speculation that will require additional studies (e.g., *in situ* hybridization). Furthermore, electrophysiological recordings will be necessary to assess patterns of activity and receptive field size of cortical neurons influenced by GABA-mediated inhibition under the same conditions. Since the changes we have described are presumably transient [normal gait is recovered after several weeks (Fox et al., 1993, 1994)], it would be important to investigate changes in GABA-IR during the recovery process and to assess whether these alterations may become irreversible given a sufficiently long period of hindlimb unloading.

An essential difference between HLS and sensory deprivation by surgical means is that the former alters GABA-IR in the somatosensory cortex through non-invasive unloading of weight-bearing muscles. For example, Warren et al. (1989) reported a 16% decrease of glutamic acid decarboxylase (GAD) immunoreactive cells in layer IV of the rat hindlimb somatosensory cortex 2 weeks after transection of the sciatic nerve. In experiments conducted in monkey visual cortex after 2–3 weeks of eye enucleation, Hendry and Jones (1986) found a 45% reduction of GABA-IR cells in layer IV. In the same region, these investigators also showed a 36% decrease of GABA-IR cells 11 weeks after eyelid suture.

We believe that HLS generates a more "realistic" chain of events than surgical deafferentation since the afferent input is not interrupted but rather significantly disrupted by the environmental manipulation. Our results suggest that non-invasive manipulations of the neuromuscular system (e.g., hindlimb suspension, alterations in gravitational forces as in spaceflight or hypergravity) can have significant effects on cortical circuitry. Other lines of work based on non-invasive procedures support this possibility, e.g., learning of new movements, compensation of rearranged movements, limb positioning effects (see Jenkins et al., 1990; Merzenich et al., 1990; Sanes et al., 1992). Since severely disruptive surgical interventions are rare but the central nervous system must constantly adjust movements in response to altered environmental conditions, we believe that studies in intact animals should be pursued to help clarify the mechanisms of cortical plasticity and adaptation under natural conditions.

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REFERENCES

- Akhtar ND, Land PW (1991): Activity-dependent regulation of glutamic acid decarboxylase in the rat barrel cortex: Effects of neonatal versus adult sensory deprivation. *J Comp Neurol* 307: 200–213.
- Cope TC, Bonasera SJ, Nichols TR (1994): Reinnervated muscles fail to produce stretch reflexes. *J Neurophysiol* 71:817–820.
- D'Amelio F, Dauntton NG (1992): Effects of spaceflight in the adductor longus muscle of rats flown in the Soviet Biosatellite Cosmos 2044: A study employing neural cell adhesion molecule (N-CAM) immunocytochemistry and conventional morphological techniques (light and electron microscopy). *J Neuropath Exp Neurol* 51:415–431.
- D'Amelio F, Dauntton NG, Fast T, Grindeland R (1987): Preliminary findings in the neuromuscular junctions of the soleus muscle of adult rats subjected to simulated weightlessness. Light and electron microscopy, *Space Life Sciences Symposium: Three Decades of Life Science Research in Space*, Washington, D.C. pp. 204–205.
- De Ryck M, Van Reempts J, Duytschaever H, Van Deuren B, Clincke G (1992): Neocortical localization of tactile/proprioceptive limb placing reactions in the rat. *Brain Res* 573:44–60.
- Dykes RW, Landry P, Metherate R, Hicks TP (1984): Functional role of GABA in cat primary somatosensory cortex: Shaping receptive fields of cortical neurons. *J Neurophysiol* 52:1066–1093.
- Edgerton VR, Roy RR (1994): Neuromuscular adaptation to actual and simulated spaceflight. In Fregly MJ, Blatteis CM (eds): "APS Handbook of Physiology, Section 4, Vol. 1 Adaptation to the Environment." New York: Oxford University Press, pp. 721–763.

- Fox RA, Corcoran M, Dauntan NG, Morey-Holton E (1994): Effects of spaceflight and hindlimb suspension on the posture and gait of rats. In: Taguchi K, Igarashi M, Mori S (eds): "Vestibular and Neural Front." Amsterdam: Elsevier, pp 603-606.
- Fox RA, Dauntan NG, Corcoran ML, Wu LC, D'Amelio F (1993): Tail suspension with and without hindlimb unloading affects neuromuscular function in the adult rat. *Neurosci Abstr* 19:147.
- Hall RD, Lindholm EP (1974): Organization of motor and somatosensory neocortex in the albino rat. *Brain Res* 66:23-38.
- Hendry SHC, Jones EG (1986): Reduction in number of immunostained GABAergic neurons in deprived-eye dominance columns of monkey area 17. *Nature* 320:750-753.
- Hicks TP, Dykes RW (1983): Receptive field size for certain neurons in primary somatosensory cortex is determined by GABA-mediated intracortical inhibition. *Brain Res* 274:160-164.
- Houser CR, Vaughn JE, Hendry SHC, Jones EG, Peters A (1984): GABA neurons in the cerebral cortex. In Jones EG, Peters A (eds): "Cerebral Cortex. Functional Properties of Cortical Cells, Vol. 2." New York: Plenum Press, pp 63-89.
- Hummelsheim H, Wiesendanger M (1985): Is the hindlimb representation of the rat's cortex a 'sensorimotor amalgam'? *Brain Res* 346:75-81.
- Ilyin EA, Oganov VS (1989): Microgravity and musculoskeletal system of mammals. *Adv Space Res* 9:11-19.
- Jacobs KM, Donoghue JP (1991): Reshaping the cortical motor map by unmasking latent intracortical connections. *Science* 251:944-947.
- Jenkins WM, Merzenich MM, Ochs MT, Allard TT, Guic-Robles E (1990): Functional reorganization of primary somatosensory cortex in adult owl monkeys after behaviorally controlled tactile stimulation. *J Neurophysiol* 63:82-104.
- Jones EG (1990): The role of afferent activity in the maintenance of primate neocortical function. *J Exp Biol* 153:155-176.
- Jones EG (1993): GABAergic neurons and their role in cortical plasticity in primates. *Cerebr Cortex* 3:361-372.
- Landgren S, Silfvenius H (1969): Projection to cerebral cortex of group I muscle afferents from the cat's hind limb. *J Physiol* 200:353-372.
- Landgren S, Silfvenius H (1971): Nucleus Z, the medullary relay in the projection path to the cerebral cortex of group I muscle afferents from the cat's hindlimb. *J Physiol* 218:551-571.
- Mathews PB (1991): The human stretch reflex and the motor cortex. *Trends Neurosci* 14:87-91.
- McIntyre AK (1974): Central actions of impulses in muscle afferent fibers. In Barker D, Hunt CC, McIntyre AK (eds): "Muscle Receptors." New York: Springer, pp 235-288.
- Merzenich MM, Recanzone GH, Jenkins WM, Nudo RJ (1990): How the brain functionally rewires itself. In: Arbib MA, Robinson JA (eds): "Natural and Artificial Parallel Computation." Cambridge, MA: MIT Press, pp 177-210.
- Nichols TR (1989): The organization of heterogenic reflexes among muscles crossing the ankle joint in the decerebrate cat. *J Physiol Lond* 410:463-477.
- Paxinos G, Watson C (1986): "The Rat Brain in Stereotaxic Coordinates." New York: Academic Press.
- Ribak CE (1978): Aspinous and sparsely-spinous stellate neurons in the visual cortex of rats contain glutamic acid decarboxylase. *J Neurocytol* 7:461-478.
- Sanes JN, Wang J, Donoghue JP (1992): Immediate and delayed changes of rat motor cortical output representation with new forelimb configurations. *Cerebr Cortex* 2:141-152.
- Thomason DB, Booth FW (1990): Atrophy of the soleus muscle by hindlimb unweighting. *J Appl Physiol* 68:1-12.
- Warren R, Tremblay N, Dykes RW (1989): Quantitative study of glutamic acid decarboxylase-immunoreactive neurons and cytochrome oxidase activity in normal and partially deafferented rat hindlimb somatosensory cortex. *J Comp Neurol* 288:583-592.
- White E (1989): "Cortical Circuits. Synaptic Organization of the Cerebral Cortex. Structure, Function and Theory." Boston: Birkhäuser.
- Wiesendanger M, Miles TS (1982): Ascending pathways of low threshold muscle afferents to the cerebral cortex and its possible role in motor control. *Physiol Rev* 62:1234-1270.
- Wronski TJ, Holton EM (1987): Skeletal response to simulated weightlessness: A comparison of suspension techniques. *Aviation Space Environ Med* 58:63-68.
- Zilles K, Wree A (1985): Cortex: Areal and laminar structure. In G. Paxinos (ed): "The Rat Nervous System, Vol. 1." New York: Academic Press, pp 375-415.

Hypergravity Exposure Decreases γ -Aminobutyric Acid Immunoreactivity in Axon Terminals Contacting Pyramidal Cells in the Rat Somatosensory Cortex: A Quantitative Immunocytochemical Image Analysis

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Quantitative evaluation of γ -aminobutyric acid immunoreactivity (GABA-IR) in the hindlimb representation of the rat somatosensory cortex after 14 days of exposure to hypergravity (hyper-G) was conducted by using computer-assisted image processing. The area of GABA-IR axosomatic terminals apposed to pyramidal cells of cortical layer V was reduced in rats exposed to hyper-G compared with control rats, which were exposed either to rotation alone or to vivarium conditions. Based on previous immunocytochemical and behavioral studies, we suggest that this reduction is due to changes in sensory feedback information from muscle receptors. Consequently, priorities for muscle recruitment are altered at the cortical level, and a new pattern of muscle activity is thus generated. It is proposed that the reduction observed in GABA-IR of the terminal area around pyramidal neurons is the immunocytochemical expression of changes in the activity of GABAergic cells that participate in reprogramming motor outputs to achieve effective movement control in response to alterations in the afferent information. *J. Neurosci. Res.* 53:135–142, 1998.

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Key words: γ -aminobutyric acid; inhibition; hindlimb; centrifugation; muscle receptors; afferent input; NIH-Image

INTRODUCTION

The afference-reafference concept (for review, see Nelson, 1996) assigns an important role to afferent input during adaptation of motor behavior to new environmental conditions. Behavioral studies have demonstrated the importance of active movement for the development of new, effective motor programs when subjects are exposed

chronically to new conditions, but the neural mechanisms that accomplish this adaptation are specified only partially. Air righting during free fall, locomotion, and limb movements during swimming are altered following chronic exposure to hypergravity (hyper-G; Fox et al., 1992). These effects are thought to reflect adaptation to the new gravitational condition and presumably are produced, in part, by a modified afferent input to the central nervous system.

One possible neurochemical component of the motor adaptation process may involve the neurotransmitter γ -aminobutyric acid (GABA). GABA immunoreactivity (GABA-IR) as well as glutamic acid decarboxylase immunoreactivity of local circuit neurons of the cerebral cortex are greatly reduced following the surgical abolition of afferent input (Hendry and Jones, 1986; Warren et al., 1989; Akhtar and Land, 1991; see also Jones, 1990). In addition, afferent activity has been shown to regulate GABA synthesis, and several lines of research have established that GABA-mediated intracortical inhibition specifies the size and thresholds of receptive fields of major neuronal subgroups (Hicks and Dykes, 1983; Dykes et al., 1984; Hendry and Jones, 1988; Jones, 1990, 1993).

We have recently shown that GABA-IR is reduced in local circuit interneurons and terminals of the rat hindlimb somatosensory cortex following 14 days of

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hindlimb unloading by tail suspension (D'Amelio et al., 1996). This procedure simulates some of the effects of weightlessness on antigravity muscles (e.g., soleus atrophy) that are observed following space flight (for reviews, see Ilyin and Oganov, 1989; Thomason and Booth, 1990; Edgerton and Roy, 1994) and causes major alterations in motor control (Corcoran et al., 1994; Fox et al., 1994). In combination, these findings suggest the involvement of GABAergic activity in neural plasticity produced by a treatment that is noninvasive to the CNS.

The goal of this experiment was to determine whether GABA-IR is altered as a result of chronic exposure to three times the normal gravity of earth (i.e., hyper-G). The hindlimb sensorimotor cortical representation was chosen for study due to its unique cytoarchitectonic organization. At this level, electrophysiological studies suggest an overlap (coextension) of sensory and motor areas ("sensorimotor amalgam"), where layer IV is entirely granular, and layer V contains large pyramidal cells that extend over, without interruption, from the motor cortex. This type of cortical organization would seem to lend support to the hypothesis first proposed by Phillips (1969) that information from muscle spindles to the cerebral cortex is relayed through an oligosynaptic transcortical spindle circuit for proprioceptive signals whose efferent limb is the corticomotoneuronal projection (Lende, 1963; Hall and Lindholm, 1974; Hummelsheim and Wiesendanger, 1985). Furthermore, investigations by De Ryck et al. (1992) have shown that the cortical substrate subserving tactile and proprioceptive limb placing coincide with a dense subfield of large pyramidal neurons in the deeper part of layer V at the level of the frontal lateral agranular and the parietal forelimb and hindlimb areas.

MATERIALS AND METHODS

Animals

Twelve young adult male rats (200–220 grams) of the Sprague-Dawley strain (Simonsen, Gilroy, CA) were used. They were assigned randomly to one of three groups ($n = 4/\text{group}$), hyper-G (exposed to centrifugation), stationary controls (VIV), and rotation controls (RC; tissue from one rat in the RC condition was discarded due to deficient perfusion). The rats were housed in pairs in shoe box style plastic cages and maintained on standard laboratory rodent chow. Access to food and water was ad libitum throughout the experiment.

Experimental Procedures

Exposure to hyper-G. Hyper-G was produced by using the 24-foot-diameter centrifuge at NASA-Ames Research Center, Moffett Field, CA. This facility has

been used extensively in studies of the physiological effects of hyper-G. The centrifuge has ten radial arms with a maximum effective operating radius of 12 feet. Holding chambers on the centrifuge are gimballed, so they swing out during rotation, ensuring that the resultant gravitational forces experienced by the animals are in the normal direction (dorsoventral).

To ensure acclimation to housing conditions, the animals were housed in standard plastic cages placed inside the holding chambers for 4 days prior to beginning the treatment conditions. Following this period of acclimation, rats in the experimental group were exposed to hyper-G treatment. The centrifuge was rotated for 14 days at 25 rpm to produce a resultant force of 3-G at the 12-foot radius where the holding chambers were located. The centrifuge was stopped four times for 30 min (there was a 2-day minimum between stops) during the 14-day period for cleaning cages, replenishing food and water, and weighing the rats. The holding chambers were lighted by fluorescent lights on an automatically controlled 12:12 hr. light:dark cycle.

Treatment of control groups. Rats in the RC group were housed in gimballed cages on the centrifuge but very near the axis of rotation (at a 1.7-foot radius). These animals experienced the angular velocity of centrifugation with only a minimal (0.06 G) increase in G force. This control group was used to assess the effects of angular velocity and Coriolis produced by movement with a minimal increase in G.

Rats in the VIV (stationary) group were housed in the centrifuge room under conditions similar to those of the rats exposed to hyper-G or rotation (i.e., caging, lighting, temperature, humidity, acoustic noise, etc.). These rats did not experience either rotation or altered G.

Tissue processing. After 14 days of centrifugation, the animals and their controls were deeply anesthetized with Metophane (Pitman-Moore, Inc., Washington Crossing, NJ) and perfused through the heart with 50 ml 0.9% saline followed by 500 ml of a fixative made up of 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed the same day, immersed in fresh fixative, and stored at 4°C. The right hemisphere was blocked coronally between Bregma -1.8 mm and Bregma -3.6 mm, where the somatosensory representation of the hindlimb is conspicuous and is associated with the presence of the rostral hippocampus (Paxinos and Watson, 1986). For identification purposes, the hemisphere of the VIV group was marked with a hole at the level of the striatum, and the hemisphere of the RC group was with two holes at the same level. Tissue sectioning and immunostaining were performed according to procedures described elsewhere (D'Amelio et al., 1996). Free-floating tissue sections of centrifuged, vivarium, and rotational animals were pro-

cessed together in the same solutions for consistent immunostaining and then placed as a group of three sections (staining triplets) on the same glass slide to comprise a staining triplet.

Quantitative Analysis

Complete details of the image-analysis procedure employed for quantitative purposes have been published elsewhere (Wu et al., 1997). Pyramidal neurons were selected when they possessed oval contours and a distinct apical dendrite. No GABA-IR product was present in the somata of these cells. Briefly, the basic equipment consisted of a Macintosh Centris 650 computer (Apple Computers, Cupertino, CA) with Scion Technology's LG-3 frame grabber (Friederick, MD.), a light microscope (Leitz Diaplan, Wetzlar, Germany) equipped with a $\times 100$ oil-immersion objective for observation of the sections, and a Sierra model 4030 CCD camera (Sunnyvale, CA.) mounted on top of the microscope. A public-domain software, NIH-Image version 1.59 (NIH, Bethesda, MD; written by Wayne Rasband and updated frequently), was employed to capture images of pyramidal neurons of layer Vb of the hindlimb representation of the somatosensory cortex and to analyze the GABA-IR terminals apposed to them (this software is available electronically from zipper.nih.gov/pub/nih-image/nih-image or from the NIH web site: <http://rsb.info.nih.gov/nih-image>). Fast Fourier Transform (FFT) routines incorporated into this version enabled us to enhance the terminals, making them easily differentiated from the cell background. Essentially, after FFT processing, the stronger signals of GABA⁺ terminals in focus became prominent, with weaker signals of negatively stained somata being reduced. A thresholding operation left only the terminals visible. Total numbers of pixels representing terminal areas were analyzed from the binary images, and the perimeter length of the soma was analyzed from the gray-scale images. The ratio of terminal area to the soma perimeter of a cell was used as a normalized value to evaluate changes in GABA-IR between the experimental and the control groups.

RESULTS

The hindlimb representation of the somatosensory cortex was identified in Nissl-stained slides by

the prominent aggregation of granular cells in layer IV, decreased cell density in layer Va, and the presence of large pyramidal cells in layer Vb (Fig. 1). Our analysis was focused on GABA-IR terminals that were

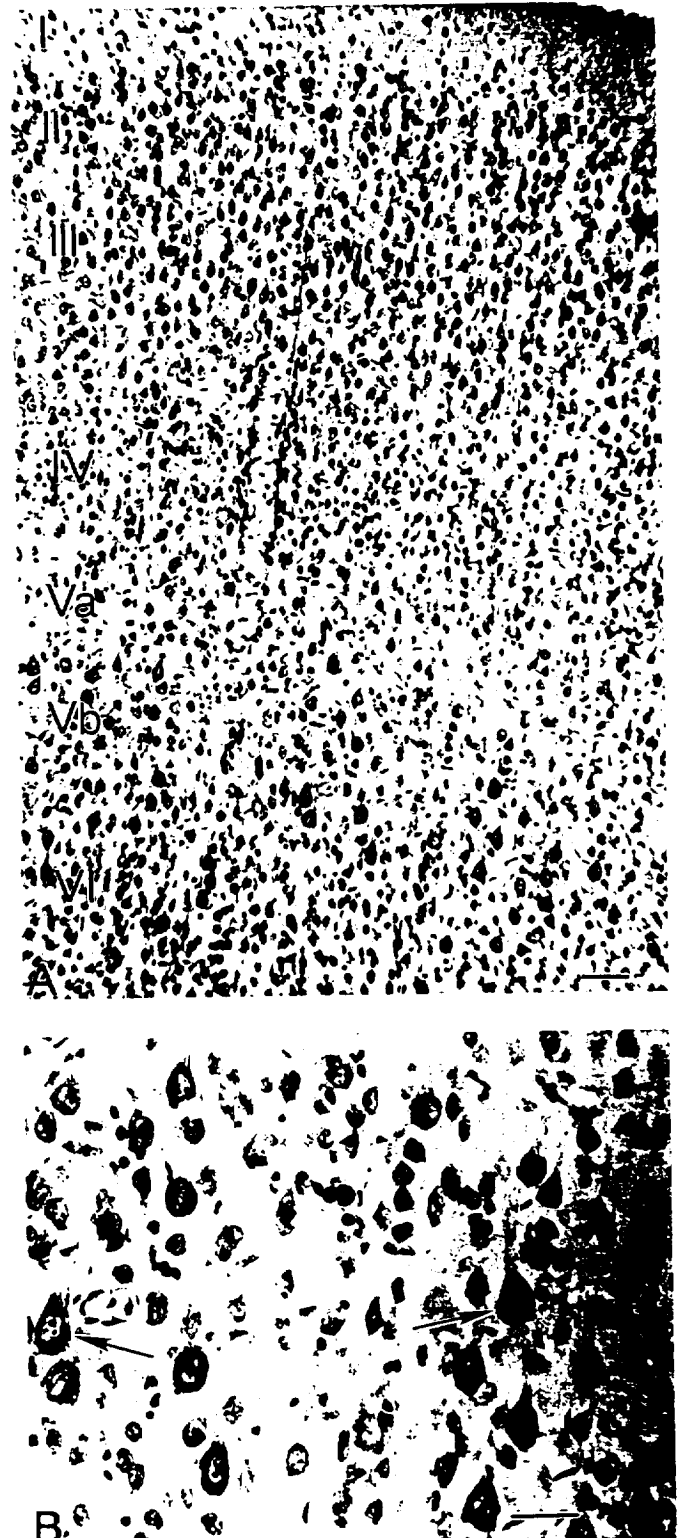


Fig. 1. **A:** Photomicrograph of a Nissl-stained coronal section through the hindlimb somatosensory cortex. Note the aggregation of granular cells in layer IV and the paucicellular layer Va. **B:** Higher magnification of layer Vb showing large pyramidal cells (arrows) of the type employed to quantify immunoreactive γ -aminobutyric acidergic (GABAergic) terminals apposed to them. Scale bars = 100 μ m in A, 50 μ m in B.

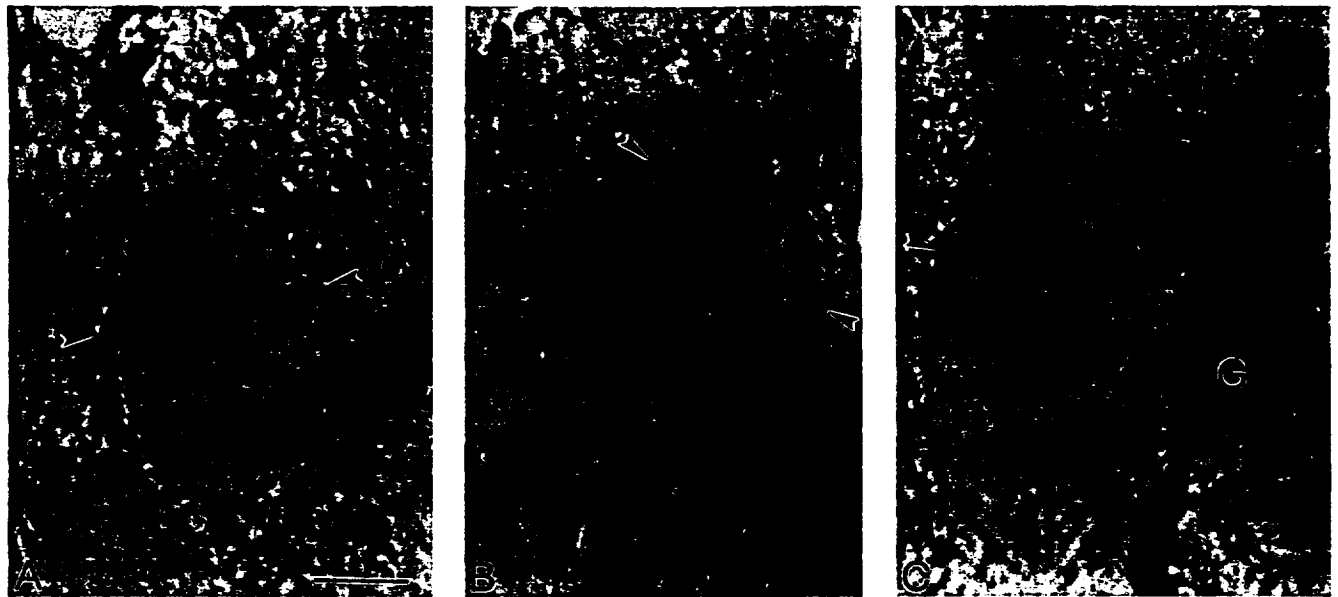


Fig. 2. Photomicrographs of sections immunostained with GABA antiserum at the level of layer Vb of the hindlimb somatosensory cortex. Three pyramidal cells are shown: vivarium control (A), rotation control (B), and centrifuged (C). The cells are crowned by GABA-immunoreactive terminals (arrowheads). Note the paucity of terminals in C. G, GABAergic cell. Scale bar = 25 μ m.

closely apposed to the pyramidal cells of layer Vb (Fig. 2).

Up to eight cells (mean = 7, range 3–8) were identified on each section. A normalized estimate of terminal area on each cell was obtained as the ratio of the total area of all identified terminals to the perimeter of the soma of that cell. Perimeter length was corrected by disregarding the gaps where apical and/or basal dendrites were present. The mean of these values for all cells on that slide was determined. These mean values were obtained for up to five slides per animal (mean = 3.5,

range 2–5). The average value, which was used for statistical analysis, was then computed to estimate the normalized terminal area for each rat.

The normalized terminal area for each of the 11 rats is summarized in Table I. Immunoreactive terminal area was significantly less in rats subjected to hyper-G than in vivarium control rats, but the terminal area in rats subjected to the RC condition did not differ from the vivarium controls (see Fig. 3).

DISCUSSION

The findings of this report demonstrate that, following exposure to hyper-G, GABA-IR is significantly reduced in axon terminals apposed to pyramidal cells of

TABLE I. Average Ratio of Terminal Area to Perimeter of the Somata for Each of the 11 Rats Used in the Experiment*

Staining triplets	3G	VIV	RC
I	6.78 (3: 19)	9.56 (3: 18)	8.96 (3: 17)
II	5.54 (3: 23)	6.37 (3: 24)	4.88 (2: 16)
III	5.27 (5: 40)	9.41 (5: 39)	8.94 (5: 40)
IV	4.95 (3: 17)	6.16 (3: 13)	X
Mean	5.63	7.88	7.59
SD	0.80	1.86	2.35
SEM	0.40	0.93	1.36
<i>t</i> test vs. VIV	3.37	—	0.25
<i>P</i> value	<0.05	—	>0.20

*Data are presented for staining triplets of three rats in which tissue of centrifuged (3G), vivarium (VIV), and rotation (RC) control rats were immunostained concurrently and mounted on single slides. Numbers in parentheses identify the number of slides and the number of cells (slides; cells) contributing to each mean for each rat.

Fig. 3. Example of digital images of three pyramidal cells (PC) employed for quantitative analysis. A,B: Centrifuged group. C,D: Vivarium control group. E,F: Rotation control group. The photomicrographs on the left (A,C,E) depict the original captured images of the cells surrounded by GABA-immunoreactive terminals (arrows). The photomicrographs on the right (B,D,F) depict images of the cells after Fast Fourier Transform processing to demonstrate the selected terminal area (dashed outlines) that was measured. There is a noticeable difference between the terminal area around the pyramidal cell of the centrifuged group and the terminal areas from the vivarium and rotation groups.

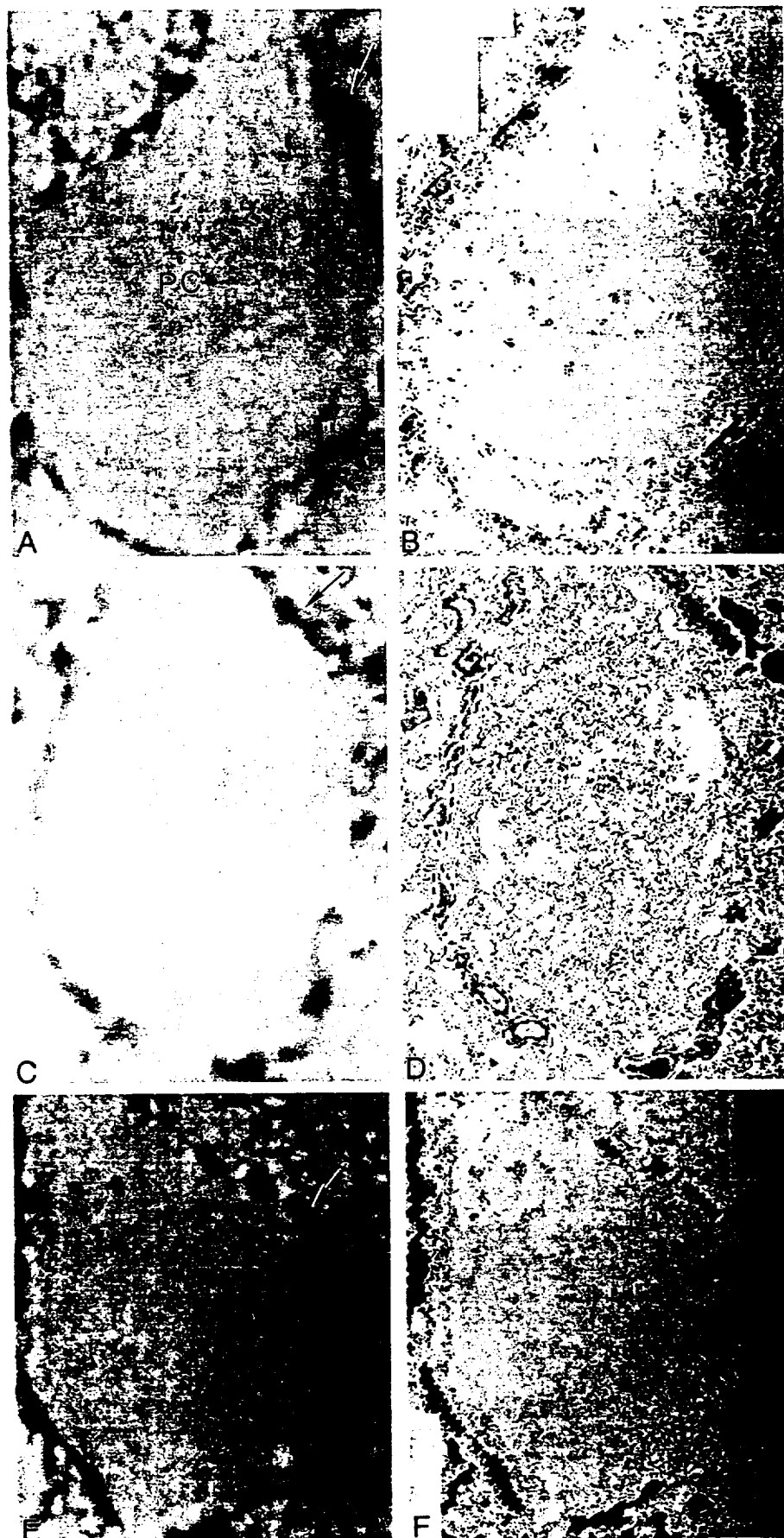


Figure 3.

the rat somatosensory cortex. Overlapping of values for animals seen in some instances apparently is due to individual deviations. We expect that the responses to noninvasive procedures used in our experiments are probably more variable than those to invasive (e.g., surgical deafferentation) procedures, in which the experimental impact is more severe. Thus, we assessed treatment effects from the pooled data (mean) of each group.

The present findings as well as those from our previous research (D'Amelio et al., 1996) suggest that cortical circuitry in regions related to proprioceptive input from muscle receptors is susceptible to noninvasive experimental manipulations. Chronic exposure to hyper-G by means of centrifugation constitutes a novel environmental condition that produces changes in behavioral and molecular events brought about by new information processed in specific areas of the CNS. It is our suggestion that the modifications in muscle activity that are required during hyper-G alter the afferent input from muscle receptors, which, in turn, affects the processing of information in the somatosensory cortex.

It is possible that our findings may represent the immunocytochemical expression of a critical role played by GABAergic cells to limit the activity of groups of pyramidal neurons in order to suppress excessive excitatory drive as a mechanism of readjustment (see Jacobs and Donoghue, 1991; see also Roberts, 1986, 1990). GABAergic cells would thus control degrees of inhibitory and disinhibitory influences in order to prevent chaotic behavior of output pyramidal neurons by creating barriers to the perturbations originated by a *new* proprioceptive input (see Roberts, 1986). It is suggested that these changes in proprioceptive signals elicit circuit modifications at the cortical level, i.e., updating of the motor program in response to the new information. Sanes et al. (1992) have noted that sensory feedback from muscle receptors to the cerebral cortex may play a fundamental role in shaping the functional organization of the motor output and its influence on the somatic musculature. We believe that, under conditions of altered G, similar mechanisms are brought into play, and a new motor (adaptive) response is generated through circuit modifications in response to proprioceptive feedback. Thus, in the new environment, the requirements for reprogramming of motor outputs to achieve the proper muscle combination may, in fact, lead to constant plastic modifications of the circuitry at the cortical level.

Jones (1990) has indicated that cortical changes occur so rapidly that they hardly depend on the formation of new connections or axon sprouting; instead, they depend on the patterns of neural activity arising from the periphery. Furthermore, the CNS output may not recruit the same muscles consistently to accomplish a pattern of movement, and muscles that act synergetically may not

always be coactive to achieve a specific movement (Buchanan et al., 1986). Latash and Anson (1996) suggest that the specific combination of muscles that should be brought into play for a required movement may not have a unique solution. Therefore, it is reasonable to assume that the nervous system has to continually evaluate recruitment priorities to respond to tasks that, although they are invariant in outcome, may not always be carried out by the same muscle combinations (see Buchanan et al., 1986).

There is evidence that GABAergic inhibition is involved in the generation of spatiotemporal patterns of muscle activity and that it also contributes to the improvement of directionality index in the motor cortex to control the activity of target muscles (Matsumura et al., 1991, 1992). Thus, as a consequence of hyper-G, synaptic inhibition mediated by GABAergic neurons may contribute to a *change of priorities for muscle recruitment at the cortical level to implement new central strategies for achieving effective movement outcome through the selection of different muscle groups* (see Latash and Anson, 1996).

Behavioral studies conducted in our laboratory indicate changes in gait after exposure to hyper-G. Animals exposed to 2-G walked more slowly than off-centrifuge control animals and adopted a form of locomotion that resulted in increased four-footed stance, perhaps to increase postural stability. In addition, normal stride sequencing and the normal relationship between stance duration and walking speed were disrupted in animals exposed to 2-G for 16 days. These data suggest that different strategies for control of locomotion are used following exposure to altered G (Fox et al., 1991) and that these strategies, although they are different or are seemingly abnormal compared with control animals, effectively produce a purposeful movement outcome, e.g., swimming, walking. We believe that, under hyper-G conditions, pyramidal cell activity—the highest central level for the recruitment of muscle groups—is altered in response to the new sensory information and that this change occurs, in part, due to the modulatory influence of local circuit GABAergic cells whose main synaptic targets are the pyramidal neurons (White, 1989; see Salin and Prince, 1996).

Under our noninvasive experimental conditions, we cannot be certain whether the decrease in GABA-IR terminals observed in the situations of limb unloading at 1-G (D'Amelio et al., 1996) and hyper-G at 3-G reflects alterations in the synthesis of the transmitter or depletion due to increased release. Resolution of these questions would require the assessment of modifications at the level of gene expression or receptor-mediated synaptic events. However, regardless of what the answers to these questions turn out to be, they will not detract from the fact

that, in both situations, i.e., limb unloading at 1-G or centrifugation at 3-G, the animal is exposed to an *environmental novelty*. Thus, the decrease in GABA-IR seen in both situations suggests that inhibitory influences in the CNS respond as a basic adaptive mechanism to adjust central motor control programs in conditions of altered gravity. In brief, in our view, environmental novelty constitutes the principal stimulus, taking precedence over physical differences in gravitational forces.

On the other hand, exposure to rotation seems to have no effect either on cortical GABA-IR or on the performance of behavioral tasks. For example, swimming and righting ability of rats subjected to 2-G centrifugation showed significant impairment with respect to rotational and vivarium control animals. No significant difference was found between the two latter groups for the same tasks (Corcoran et al., 1991).

Despite the fact that much of what has been expressed in the foregoing is necessarily speculative, we believe that it provides an approach to the understanding of modifications of neurotransmitter behavior in the CNS when sensory motor programs are altered by environmental, i.e., noninvasive, manipulations. Most of the studies on neuroanatomical or neurochemical modifications in the CNS, as pointed out above, are based on sensory deprivation by surgical means (see, e.g., Hendry and Jones, 1986; Warren et al., 1989; Hendry et al., 1994). Although it seems obvious, it is important to stress the fact that surgical deafferentation experiments are concerned with effects provoked by the *abolition of information* from afferent sources, whereas noninvasive modifications of the afferent input, e.g., changes in gravitational forces, are concerned with the effects of an *altered inflow of information* and with the significance of afferent feedback on cortical plasticity.

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REFERENCES

- Akhtar ND, Land PW (1991): Activity-dependent regulation of glutamic acid decarboxylase in the rat barrel cortex: Effects of neonatal versus adult sensory deprivation. *J Comp Neurol* 307:200-213.
- Buchanan TS, Almdale DPJ, Lewis JL, Zev Rymer W (1986): Characteristics of synergic relations during isometric contractions of human elbow muscles. *J Neurophysiol* 56:1225-1241.
- Corcoran ML, Dauntong NG, Fox RA, Wu LC (1991): Gravitational rather than rotational component of centrifugation affects vestibular and locomotor function in the rat. *ASGSB Bulletin* 5:42.
- Corcoran ML, Fox RA, Dauntong NG, Morey-Holton E (1994): Spaceflight and Hindlimb Suspension Affect the Posture and Gait of Rats. Research and Technology Report 1994. Moffett Field, CA: NASA-Ames Research Center, NASA TM-108858, pp 163-164.
- D'Amelio F, Fox RA, Wu LC, Dauntong NG (1996): Quantitative changes of GABA-immunoreactive cells in the hindlimb representation of the rat somatosensory cortex after 14-day hindlimb unloading by tail suspension. *J Neurosci Res* 44:532-539.
- De Ryck M, Van Reempts J, Duytschaever H, Van Deuren B, Clinck G (1992): Neocortical localization of tactile/proprioceptive limb placing reactions in the rat. *Brain Res* 573:44-60.
- Dykes RW, Landry P, Metherate R, Hicks TP (1984): Functional role of GABA in cat primary somatosensory cortex: Shaping receptive fields of cortical neurons. *J Neurophysiol* 52:1066-1093.
- Edgerton VR, Roy RR (1994): Neuromuscular adaptation to actual and simulated spaceflight. In Fregly MJ, Blatteis CM (eds): "APS Handbook of Physiology, Section 4, Adaptation to the Environment." New York: Oxford University Press, pp 721-763.
- Fox RA, Dauntong NG, Wu LC, Corcoran ML (1991): Effects of chronic hyper-gravity on gait in the rat. Third IBRO World Congress of Neuroscience, Montreal, August 1991. *IBRO Abstr* 3:84.
- Fox RA, Dauntong NG, Corcoran ML, Wu LC (1992): Time-course of behavioral adaptation to hyper-gravity. In Woolacott M, Houk F (eds): "Posture and Gait: Control Mechanisms." Portland: University of Oregon Books, pp 178-181.
- Fox RA, Corcoran M, Dauntong NG, Morey-Holton E (1994): Effects of spaceflight and hindlimb suspension on the posture and gait of rats. In Taguchi K, Igarashi M, Mori S (eds): "Vestibular and Neural Front." Amsterdam: Elsevier, pp 603-606.
- Hall RD, Lindholm EP (1974): Organization of motor and somatosensory neocortex in the albino rat. *Brain Res* 66:23-38.
- Hendry SHC, Jones EG (1986): Reduction in number of immunostained GABAergic neurons in deprived-eye dominance columns of monkey area 17. *Nature* 320:750-753.
- Hendry SHC, Jones EG (1988): Activity-dependent regulation of GABA expression in the visual cortex of adult monkeys. *Neuron* 1:701-712.
- Hendry SHC, Huntsman M-M, Viñuela A, Möhler H, de Blas AL, Jones EG (1994): GABA_A receptor subunit immunoreactivity in primate visual cortex: Distribution in macaques and humans and regulation by visual input in adulthood. *J Neurosci* 14:2383-2401.
- Hicks TP, Dykes RW (1983): Receptive field size for certain neurons in primary somatosensory cortex is determined by GABA-mediated intracortical inhibition. *Brain Res* 274:160-164.
- Hummelsheim H, Wiesendanger M (1985): Is the hindlimb representation of the rat's cortex a sensorimotor amalgam? *Brain Res* 346:75-81.
- Ilyin EA, Oganov VS (1989): Microgravity and musculoskeletal system of mammals. *Adv Space Res* 9:11-19.
- Jacobs KM, Donoghue JP (1991): Reshaping the cortical motor map by unmasking latent intracortical connections. *Science* 251:944-947.
- Jones EG (1990): The role of afferent activity in the maintenance of primate neocortical function. *J Exp Biol* 153:155-176.
- Jones EG (1993): GABAergic neurons and their role in cortical plasticity in primates. *Cerebral Cortex* 3:361-372.
- Latash ML, Anson JG (1996): What are "normal movements" in atypical populations? *Behav Brain Sci* 19:55-106.
- Lende RA (1963): Cerebral cortex: A sensorimotor amalgam in marsupialia. *Science* 141:730-732.

- Matsumura M, Sawaguchi T, Oishi T, Ueki K, Kubota K (1991): Behavioral deficits induced by local injection of bicuculline and muscimol into the primate motor cortex. *J Neurophysiol* 65: 1542–1553.
- Matsumura M, Sawaguchi T, Kubota K (1992): GABAergic inhibition of neuronal activity in the primate motor and premotor cortex during voluntary movement. *J Neurophysiol* 68:692–702.
- Nelson RJ (1996): Interactions between motor commands and somatic perception in sensorimotor cortex. *Curr Opin Neurobiol* 6:801–810.
- Paxinos G, Watson C (1986): "The Rat Brain in Stereotaxic Coordinates." New York: Academic Press.
- Phillips CG (1969): The Ferrier Lecture 1968. Motor apparatus of the baboon's hand. *Proc. R. Soc. London B* 173:141–174.
- Roberts E (1986): What do GABA neurons really do? They make possible variability generation in relation to demand. *Exp Neurol* 93:279–290.
- Roberts E (1990): A systems approach to aging, Alzheimer's disease, and spinal cord regeneration. *Progr Brain Res* 86:339–355.
- Salin PA, Prince DA (1996): Spontaneous GABA_A receptor-mediated inhibitory currents in adult rat somatosensory cortex. *J Neurophysiol* 75:1573–1588.
- Sanes JN, Wang J, Donoghue JP (1992): Immediate and delayed changes of rat motor cortical output representation with new forelimb configurations. *Cerebral Cortex* 2:141–152.
- Thomason DB, Booth FW (1990): Atrophy of the soleus muscle by hindlimb unweighting. *J Appl Physiol* 68:1–12.
- Warren R, Tremblay N, Dykes RW (1989): Quantitative study of glutamic acid decarboxylase-immunoreactive neurons and cytochrome oxidase activity in normal and partially deafferented rat hindlimb somatosensory cortex. *J Comp Neurol* 288:583–592.
- White E (1989): "Cortical Circuits. Synaptic Organization of the Cerebral Cortex. Structure, Function and Theory." Boston: Birkhäuser.
- Wu LC, D'Amelio F, Fox RA, Polyakov I, Dauntan NG (1997): Light microscopic image analysis system to quantify immunoreactive terminal area apposed to nerve cells. *J Neurosci Methods* 74:89–96.

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Chronic Exposure to Hypergravity Affects Thyrotropin-Releasing Hormone Levels in Rat Brainstem and Cerebellum

Abstract

In studies to determine the neurochemical mechanisms underlying adaptation to altered gravity we have investigated changes in neuropeptide levels in brainstem, cerebellum, hypothalamus, striatum, hippocampus, and cerebral cortex by radioimmunoassay. Fourteen days of hypergravity (hyperG) exposure resulted in significant increases in thyrotropin-releasing hormone (TRH) content of brainstem and cerebellum, but no changes in levels of other neuropeptides (β -endorphin, cholecystokinin, met-enkephalin, somatostatin, and substance P) examined in these areas were found, nor were TRH levels significantly changed in any other brain regions investigated. The increase in TRH in brainstem and cerebellum was not seen in animals exposed only to the rotational component of centrifugation, suggesting that this increase was elicited by the alteration in the gravitational environment. The only other neuropeptide affected by chronic hyperG exposure was met-enkephalin, which was significantly decreased in the cerebral cortex. However, this alteration in met-enkephalin was found in both hyperG and rotation control animals and thus may be due to the rotational rather than the hyperG component of centrifugation. Thus it does not appear as if there is a generalized neuropeptide response to chronic hyperG following 2 weeks of exposure. Rather, there is an increase only of TRH and that occurs only in areas of the brain known to be heavily involved with vestibular inputs and motor control (both voluntary and autonomic). These results suggest that TRH may play a role in adaptation to altered gravity as it does in adaptation to altered vestibular input following labyrinthectomy, and in cerebellar and vestibular control of locomotion, as seen in studies of ataxia.

Key Words

Brainstem
Cerebellum
Hypothalamus
Striatum
Cortex
Thyrotropin-releasing hormone
Met-enkephalin
Substance P
Centrifugation, chronic

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Introduction

Chronic exposure to hypergravitational (2 or 3 g) environments produced by centrifugation on a large-radius centrifuge results in alteration of vestibulospinal function (control of orientation, posture, and locomotion; suppression of otolith-spinal reflex response to free fall) which gradually returns to normal during reexposure to the normal 1 g environment [1–3]. Morphological studies conducted by Ross [4] in conjunction with the cited behavioral studies have shown that there is a reduction in numbers of synapses on type II hair cells in the utricular maculae of animals exposed to chronic hypergravity (2 g). These studies suggest that there are alterations in the processing of vestibular inputs and in the interaction of vestibular inputs with other sensory and motor circuitry as the animals adapt to hyperG and learn to move normally and effectively in the hyperG environment.

Several lines of evidence suggest that neuropeptides, particularly ACTH_(1–10), thyrotropin-releasing hormone (TRH), and substance P, may be involved in vestibular compensation following unilateral labyrinthectomy or deaf-ferentation [5–9]. Since long-term exposure to hyperG provides another situation in which vestibular inputs are chronically altered, we have evaluated whether various neuropeptides might be involved in adaptation to altered gravity as a first attempt at identifying the neural mechanisms that participate in the adaptation process. In the biochemical study reported here we collected brain tissue for assessment of changes in neuropeptide levels in various regions. We were particularly interested in the brainstem and cerebellum, since these areas are most intimately involved with vestibulospinal control of orientation, posture and locomotion which were found to be altered for up to several days following 14 days of hyperG exposure in our previous behavioral studies.

Materials and Methods

Animals

Male Sprague-Dawley rats (Simonsen, Gilroy, Calif.), aged approximately 52–55 days at the start of the 14 days of centrifugation, and weighing 270–310 g at time of tissue harvesting, were used. All rats were housed 2 per cage in identical, standard 'shoebox' cages lined with bedding, and maintained on standard rodent chow (Wayne), with free access to food and water. Lighting was provided on a 12:12-hour light:dark cycle. Light intensity was equated for all cages. Animals were randomly assigned to one of three groups: stationary control group (n = 10), 2 g group (n = 10), and rotation control group (n = 6). Animals in the rotation control group were housed near the center of rotation so that they experienced the angular velocity component of centrifugation, but not the hyperG component. A limitation of space near the axis made it possible to house only 6 animals in this group. The stationary control group, housed in the room containing the centrifuge, experienced conditions (caging, light, temperature, acoustic noise) similar to those of the centrifuged groups, but did not experience rotation or altered gravity.

Centrifugation

HyperG was provided by the Ames Research Center's '24 Foot Diameter Centrifuge'. For animals in the centrifuged groups the shoebox cages were placed into large (23.5 inches high × 39.5 inches wide × 22 inches deep; 58.8 × 78.8 × 55 cm), opaque, ventilated enclosures, suspended from the radial arms of the centrifuge by gimbaled yokes. These yokes allowed the enclosures to swing out with the centrifugal force produced by rotation of the centrifuge. The smaller enclosures (12 inches high × 21.5 inches wide × 11.5 inches deep; 30 × 53.8 × 28.8 cm) used to house the rotation control animals were similarly suspended from gimbaled yokes. Thus, during centrifugation the animals were subjected to the resultant of gravitational and centrifugal forces in the normal (dorsal-ventral) direction (i.e., perpendicular to the cage floor). The centrifuge was run at 20.5 rpm, providing a resultant force of 2 g for the animals placed in the enclosures at the maximum radius (12 ft – 2 g group) and 1.03 g for animals housed near the axis of rotation (radius = 1.7 ft; rotation control group). During the 14-day study the centrifuge was stopped for 30 min on days 4, 7, and 11 so that animals could be checked and weighed, cages cleaned, and food and water replenished.

Sample Collection and Tissue Extraction

Immediately on stopping the centrifuge at 9.30 a.m. on day 14, animals were weighed, and trunk blood and brain tissue were harvested following decapitation, with the centrifuged animals taken first (alternating 2 g and rotation control animals). Tissue from centrifuged animals was obtained between 4 and 53 min following centrifugation, while tissue from stationary control animals was collected during the following 30-min period. All tissue harvesting was completed within 1.5 h after stopping the centrifuge. The brainstem (including medulla and pons), cerebellum (including the whole cerebellum), hypothalamus, pituitary, striatum, hippocampus, and the entire cerebral cortex were quickly dissected out on ice and frozen on dry ice before storage at -70°C . Blood (approximately 1 ml) was collected in tubes, placed on ice for 30 min or less, and then centrifuged at 4°C and 2,000 rpm for 30 min. Plasma was removed and stored at -70°C . For extraction, the tissue samples were homogenized with 1.5 ml 2 N acetic acid, and boiled for 10 min. An aliquot of the extract was taken for protein determination according to the method of Lowry et al. [10] before the extract was centrifuged for 20 min at 13,000 rpm and 4°C . The supernatants were aliquoted, shipped, along with the plasma, to Hong Kong on dry ice, and lyophilized.

Radioimmunoassay

TRH, met-enkephalin, substance P, somatostatin, and cholecystokinin (CCK) were measured by radioimmunoassay (RIA) as described previously by Tang et al. [11], Tang and Man [12], and Wang et al. [13]. The antisera for substance P, somatostatin, and CCK were generous gifts from Dr. J. Hong (NIEHS, Research Triangle Park, N.C.), while the antiserum for

TRH was kindly supplied by Dr. J.S. Kizer (University of North Carolina). Met-enkephalin antiserum was purchased from Incstar (Stillwater, Minn.). The specificities of the antisera have been reported elsewhere [11–13]. The antiserum for β -endorphin was raised in Tang's laboratory and cross-reacted 70% with β -lipotropin on a molar basis. Corticosterone levels were determined by RIA using antiserum obtained from Radioassays Laboratory, following procedures described previously [14]. Samples from stationary control, 2 g, and rotation control animals were always measured simultaneously in the same assay. Protein content of tissues was determined and the concentration of each neuropeptide was expressed in pg or ng per mg protein.

Statistical Analysis

Statistical analyses were performed by ANOVA using Statview 4.0 (Abacus). Specific comparisons of values from 2 g and rotation control animals with those from stationary controls were made using Fisher's PLSD. Differences were considered to be significant when $p < 0.05$. All values were expressed as mean \pm SEM.

Results

Thyrotropin-Releasing Hormone

Table 1 shows the results for TRH measured in all brain areas investigated. There was a significant increase (+24%) in mean TRH level in the brainstem of animals exposed to 2 g, as compared with stationary con-

Table 1. Effects of hyperG on TRH levels (pg/mg protein, except for hypothalamus, which is in ng/mg protein) in various rat brain regions

	Control (n = 10)	HyperG (n = 10)	Rotation (n = 6)
Brainstem	287 ± 10	$357 \pm 25^*$	321 ± 25
Cerebellum	28 ± 2	$39 \pm 4^*$	26 ± 3
Hypothalamus	3.79 ± 0.11	3.8 ± 0.18	3.49 ± 0.22
Striatum	23.4 ± 2.26	$17.4 \pm 2.02^*$	$7.0 \pm 1.03^*$
Hippocampus	15.3 ± 5.5	31.0 ± 6.7	13.4 ± 9.0
Cortex	6.52 ± 0.73	6.04 ± 0.62	7.00 ± 0.86

* $p < 0.05$ compared with control level.

Table 2. Effects of hyperG on met-enkephalin (pg/mg protein, except for hypothalamus and striatum, which are in ng/mg protein) in various brain regions

	Control (n = 10)	HyperG (n = 10)	Rotation (n = 6)
Brainstem	2.55 ± 0.14	2.56 ± 0.07	2.56 ± 0.13
Cerebellum	754 ± 34	741 ± 29	870 ± 54
Hypothalamus	4.00 ± 0.11	3.89 ± 0.07	3.88 ± 0.11
Striatum	6.96 ± 0.35	7.71 ± 0.60	7.19 ± 0.71
Hippocampus	883 ± 32	977 ± 56	888 ± 46
Cortex	392 ± 21	319 ± 22*	314 ± 21*

* p < 0.05 compared with control level.

Table 3. Effects of hyperG on CCK (pg/mg protein) in various brain regions

	Control (n = 10)	HyperG (n = 10)	Rotation (n = 6)
Brainstem	114 ± 3.8	122 ± 6.2	138 ± 6.4*
Hypothalamus	923 ± 27	853 ± 36	905 ± 44
Striatum	1,132 ± 44	1,110 ± 54	1,135 ± 53
Hippocampus	1,100 ± 71	1,075 ± 30	1,211 ± 41
Cortex	430 ± 18	462 ± 13	473 ± 19

* p < 0.05 compared with control level.

trol animals, but not in those exposed only to the angular velocity component of centrifugation (rotation controls). There also was a significant increase (39%) in TRH levels in the cerebellum of 2 g animals. In the striatum, TRH levels were significantly decreased in both 2 g (–26%) and rotation control (–70%) animals compared with stationary controls, and the decrease was significantly larger in the rotation controls than in the 2 g animals. No significant changes were found in any other brain areas in either group of centrifuged animals.

Met-Enkephalin

The results of measurements of met-enkephalin in the various brain regions are

shown in table 2. There was a significant decrease (–19%) in met-enkephalin in the cerebral cortex of 2 g animals and a similar decrease (–20%) in rotation control animals as compared with stationary controls. No other brain areas showed significant changes in met-enkephalin levels in either 2 g or rotation control animals.

Cholecystokinin

CCK was measured in all investigated brain regions except the cerebellum. The results are shown in table 3. There were no changes in levels of this neuropeptide except in the brainstem of rotation control animals in which CCK was significantly increased (+21%).

β -Endorphin, Somatostatin, and Substance P

The levels of somatostatin and substance P were obtained from each brain region investigated, while β -endorphin was assessed only in the brainstem and hypothalamus. None of the levels for any of these neuropeptides showed significant alterations as a result of exposure to hyperG or to rotation (results not shown).

Corticosterone

Serum corticosterone was assayed to obtain a measure of stress in the animals. There was no significant difference between mean corticosterone level in 2 g and stationary control animals (3.56 ± 0.69 vs. 3.51 ± 0.72 $\mu\text{g/ml}$). However, in rotation control animals the mean corticosterone level was significantly elevated (14.95 ± 2.05 $\mu\text{g/ml}$) as compared with that in both stationary control animals and 2 g animals. The coefficient of variation for the rotation control group (0.14) is similar to that for the stationary control group (0.21), suggesting that this large increase is not the result of sampling error.

Discussion

Of the neuropeptides investigated in this experiment, only TRH showed significant changes related specifically to hyperG exposure, and not to the rotational component of centrifugation. These hyperG-related changes occurred only in brainstem and cerebellum, areas known to be involved with vestibular control of voluntary, postural, and autonomic functions. Thus, it seems likely that the increase in TRH levels in the brainstem and cerebellum may be related specifically to the process of sensory-motor adaptation to altered gravity. Since there is no change of TRH level in the hypothalamus, it is probably not the hormonal role of this peptide that is involved,

but rather the neuromodulatory role. The fact that this neuropeptide has been shown to play a role in vestibular compensation, another form of sensory-motor adaptation [6, 7] lends support to this suggestion, while the finding that TRH levels are also elevated in the brainstem and cerebellum of ataxic mice suggests that the increase of TRH in these areas may be specifically related to disturbances of postural and locomotor control [15], a situation which we have previously shown exists following chronic hyperG exposure [1, 2]. An involvement of TRH in postural and locomotor control comes as no surprise. TRH has been found useful in the treatment of other conditions in which locomotor and postural control are disrupted, i.e., ataxia due to spinocerebellar degeneration and spinal cord injury [e.g., 16, 17]. TRH receptors have been identified in the medial and spinal (descending) vestibular nuclei [18], and in dorsal and ventral horns of the spinal cord [19]. TRH causes excitation of motoneurons in the spinal cord [20, 21] and increases locomotor activity in the rat [22]. TRH also improves locomotor balance after bilateral labyrinthectomy [23], and is a neurotrophic factor in cultured spinal motor neurons [24].

A number of studies suggest that pituitary and hypothalamic peptides (especially TRH and $\text{ACTH}_{(4-10)}$) may play an important role in behavioral adaptation to the environment and in learning, while other studies show that they also facilitate vestibular compensation [5, 9, 25–27]. Other effects of TRH possibly related to adaptation include learning- and memory-enhancement [e.g., 28], effects on Purkinje cell activity [29], and facilitation of dendritic sprouting and/or synaptic efficacy because of its demonstrated influence on protein biosynthesis [30]. Other actions of TRH that might promote recovery include improvement of blood flow, reversing or attenuating physiological effects of endogenous

opioids, and attenuating the effects of platelet-activating factor [17, 20]. Alternatively, it has been suggested that TRH, as well as other drugs which facilitate vestibular compensation, do so by increasing general motor activity or arousal, thus providing more opportunity to learn new sensory-motor relationships [23].

Although the mechanism by which TRH influences postural and locomotor control in sensory-motor adaptation is not clear, one possibility involves the known interaction of TRH with dopamine receptors, producing a dopaminergic activating effect [7, 31]. The finding that D2 receptor stimulation can lead to TRH release provides an interesting clue to the dopamine/TRH interrelationship [32] and suggests further approaches to delineating the role of TRH in sensory-motor adaptation.

Levels of two neuropeptides were significantly changed in both the hyperG and the rotation control animals (i.e., TRH in the striatum and met-enkephalin in the cortex). The striatum is important in initiating movement by sending signals through the thalamus to the premotor cortex. Due to the different cross-coupling forces experienced by the rotation animals run at the short radius, the vestibular stimulation and requirements for control of locomotion and posture are quite different from those experienced by the 2 g animals run at the large radius. These differences in motor control requirements may result in the differences in striatal TRH content in the two groups of animals. In addition, the levels of one neuropeptide (i.e., CCK) were found to be significantly altered in the brainstem of the rotation control animals, but not the hyperG animals. While these alterations in neuropeptide levels in animals exposed to a rotating environment (i.e., both hyperG and rotation control animals) are certainly of general interest, they are not germane to this investigation since we are attempting to identify those neu-

ropeptides which show changes that might be related to the alterations in vestibular, postural and locomotor function seen following hyperG exposure. Since no alterations in these functions are found in rotation control animals, it is unlikely that the neuropeptide changes seen in both rotation and hyperG animals are relevant to our investigation.

Very high levels of corticosterone were detected in rotation control animals, while levels in hyperG animals were similar to those in the stationary control group. It cannot be determined from this experiment whether the rotation control animals were indeed more stressed than the hyperG animals, or alternatively, whether for some reason corticosterone levels in the hyperG animals were suppressed. One possible explanation may be suggested based on the finding of decreased sensitivity in at least the gravity-sensing portion of the vestibular system of hyperG, but not rotation control animals following chronic hyperG exposure [2, 33]. It is possible that a short-term stressful vestibular aftereffect of rotation occurs in animals with normal vestibular sensitivity (i.e., rotation control animals), but not in the hyperG animals in which vestibular sensitivity is suppressed.

While the changes seen in this biochemical study were relatively small, it is likely that with sampling of the specific subregions in the brainstem and cerebellum (e.g., vestibular nuclei, vestibulocerebellum) where the effect occurs a more dramatic response would be detected. We do not know from the present experiment specifically what areas in the brainstem and cerebellum are contributing to this significant increase in TRH levels. However, experiments are underway using both immunocytochemistry and receptor binding techniques to identify the subregions of brainstem and cerebellum that may be responsible for the increased levels of TRH found following chronic hyperG exposure.

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References

- 1 Fox R, Daunton N, Corcoran M, Wu L: Time course of behavioral adaptation to hyper-gravity: in Woolacott M, Houk F (eds): *Posture and Gait: Control Mechanisms*. Portland, University of Oregon Books, 1992, pp 178-181.
- 2 Fox R, Daunton N, Corcoran M: Study of adaptation to altered gravity through systems analysis of motor control. *Adv Space Res* 1998;22: 245-253.
- 3 Sondag E: *The Effects of Hypergravity on Function and Structure of the Peripheral Vestibular System*. Amsterdam, Ponsen & Looijen, 1996.
- 4 Ross MD: Morphological changes in rat vestibular system following weightlessness. *J Vest Res* 1993;3: 241-251.
- 5 Flohr H, Abeln W, Lüneburg U: Neurotransmitter and neuromodulator systems involved in vestibular compensation: in Berthoz A, Melvill Jones G (eds): *Adaptive Mechanisms in Gaze Control: Facts and Theories*. New York, Elsevier, 1985, pp 269-277.
- 6 Igarashi M, Thompson GC, Thompson AM, Usami S: Neurochemical and neuropharmacological studies on vestibular compensation/adaptation: in Hwang JC, Daunton NG, Wilson VJ (eds): *Basic and Applied Aspects of Vestibular Function*. Hong Kong, Hong Kong University Press, 1988, pp 89-97.
- 7 Ishii M, Igarashi M: Effect of thyrotropin releasing hormone on vestibular compensation in primates. *Am J Otolaryngol* 1986;7:111-180.
- 8 Lüneburg U, Flohr H: Effect of substance P on vestibular compensation: in Flohr H (ed): *Post-Lesion Neural Plasticity*. Berlin, Springer, 1988, pp 699-704.
- 9 Smith P, Darlington C: Can vestibular compensation be enhanced by drug treatment? A review of recent evidence. *J Vestib Res* 1994;4:169-179.
- 10 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-275.
- 11 Tang F, Cheung A, Vacca-Galloway L: Measurement of neuropeptides in the brain and spinal cord of Wobbler mouse: A model for motoneuron disease. *Brain Res* 1990;518:329-333.
- 12 Tang F, Man W: The regional distribution of thyrotropin releasing hormone, leu-enkephalin, met-enkephalin, substance P, somatostatin, and cholecystokinin in the rat brain and pituitary. *Neuropeptides* 1991;19: 287-292.
- 13 Wang ZP, Man SY, Tang F: Age-related changes in the contents of neuropeptides in the rat brain and pituitary. *Neurobiol Aging* 1993;14: 529-534.
- 14 Tang F, Wong RP: Pituitary contents of beta-endorphin, dynorphin, substance P, cholecystokinin, and somatostatin in rats with streptozotocin-induced diabetes. *Biol Signals* 1996;5:44-50.
- 15 Nakayama T, Nagai Y: Alterations in local cerebral glucose metabolism and endogenous thyrotropin-releasing hormone levels in Rolling Mouse Nagoya and effect of thyrotropin-releasing hormone tartrate. *Jpn J Pharmacol* 1996;72:241-246.
- 16 Manyam B: Recent advances in the treatment of cerebellar ataxias. *Clin Neuropharmacol* 1986;9:508-516.
- 17 Behrmann D, Bresnahan J, Beattie M: Modeling of acute spinal cord injury in the rat: Neuroprotection and enhanced recovery with methylprednisolone, U-74006F and YM-14673. *Exp Neurol* 1994;126:61-75.
- 18 Zanni M, Giardino L, Toschi L, Galetti G, Calzà L: Distribution of neurotransmitters, neuropeptides, and receptors in the vestibular nuclei complex of the rat: An immunocytochemical, in situ hybridization and quantitative receptor autoradiographic study. *Brain Res Bull* 1995; 36:443-452.
- 19 Winokur A, Manaker S, Kreider M: TRH and TRH receptors in the spinal cord. *Ann NY Acad Sci* 1989; 553:314-324.
- 20 Faden A: TRH analog YM-14673 improves outcome following traumatic brain and spinal cord injury in rats: Dose-response studies. *Brain Res* 1989;486:228-235.
- 21 Tremblay LE, Bedard PJ: Action of 5-hydroxytryptamine, substance P, thyrotropin releasing hormone and clonidine on spinal neuron excitability. *J Spinal Cord Med* 1995;18:42-46.
- 22 Clarke KA, Parker AJ: Effects of a thyrotropin releasing hormone analog on locomotor and other motor activity in the rat. *Neuropeptides* 1985;6:101-112.
- 23 Igarashi M, Ishii M, Ishikawa K, Himi T: Comparative effect of some neurotropic agents on balance compensation after unilateral and bilateral (two-staged) labyrinthectomy in squirrel monkeys: in Flohr H (ed): *Post-Lesion Neural Plasticity*. Berlin, Springer, 1988, pp 627-634.

- 24 Askanas V, Engle WK, Eagleson K, Micaglio G: Influence of TRH and TRH analogues RGH-2202 and DN-1417 on cultured ventral spinal cord neurons. *Ann NY Acad Sci* 1989;553:325-336.
- 25 De Wied D, Jolles J: Neuropeptides derived from pro-opiomelanocortin: Behavioral, physiological, and neurochemical effects. *Physiol Rev* 1982;62: 977-1059.
- 26 Dunn AJ: Effects of ACTH, beta-lipotropin, and related peptides on the central nervous system; in Nemeroff CB, Dunn AJ (eds): *Peptides, Hormones and Behavior*. New York, Spectrum, 1984, pp 273-348.
- 27 Lüneburg U, Flohr H: Effects of melanocortins on vestibular compensation. *Prog Brain Res* 1988;76:421-429.
- 28 Miyamoto M, Hirai K, Takahashi H, Kato K, Nishiyama M, Okada H, Nagaoka A: Effects of sustained release formulation of thyrotropin-releasing hormone on learning impairments caused by scopolamine and AF64A in rodents. *Eur J Pharmacol* 1993;238:181-189.
- 29 Ishii M, Igarashi M, Honda Y: Effect of TRH and DH-1417 on the vestibuloocular reflex in squirrel monkeys. *Acta Otolaryngol Suppl* 1988;447:61-66.
- 30 Lacour M, Ez-Zaher L, Xerri C: Sensorimotor activity and metabolic factors in vestibular compensation; in Flohr H (ed): *Post-Lesion Neural Plasticity*. Berlin, Springer, 1988, pp 675-685.
- 31 Mushiroy T, Shibahara R, Tamura M, Shimizu T, Itoh Y, Ukai Y, Yoshikuni Y, Kimura K: Montirelin hydrate (NS-3), a TRH analog, improved the disturbance of consciousness caused by head concussion and pentobarbital in mice. *Nippon Yakurigaku Zasshi* 1996;107: 237-245.
- 32 Przegalinski E, Jaworska L, Budziszewska B: The role of dopamine receptors in the release of thyrotropin-releasing hormone from the rat striatum and nucleus accumbens: An in vitro study. *Neuropeptides* 1993;25:277-282.
- 33 Daunton N, Fox R, Corcoran M, Taber P, Duran M, Wu L: Suppression of otolith-spinal reflex by chronic hyper-gravity exposure and by chronic streptomycin treatment. *Neurosci Abstr* 1997;23:754.

Effects of spaceflight and hindlimb suspension on the posture and gait of rats

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Introduction

Instability of posture and gait in astronauts following spaceflight (SF) is thought to result from muscle atrophy and from changes in sensory-motor integration in the CNS that occur during adaptation to micro-G. Individuals are thought to have developed, during SF, adaptive changes for the processing of proprioceptive, vestibular and visual sensory inputs [1] with reduced weighting of gravity-based signals and increased weighting of visual and tactile cues [2]. This sensory-motor "rearrangement" in the CNS apparently occurs to optimize neuromuscular system function for effective movement and postural control in micro-G. However, these adaptive changes are inappropriate for the 1g environment and lead to disruptions in posture and gait on return to Earth.

Few reports are available on the effects of SF on the motor behavior of animals. Rats studied following 18.5-19.5 days of SF in the COSMOS program were described as being "...inert, apathetic, slow" and generally unstable [3, p. 334]. The hindlimbs of these rats were "...thrust out from the body with fingers pulled apart and the shin unnaturally pronated" [3, p. 335]. On the 6th postflight day motor behavior was described as similar to that observed in preflight observations.

Improved understanding of the mechanisms leading to these changes can be obtained in animal models through detailed analysis of neural and molecular mechanisms related to gait. To begin this process the posture and gait of rats were examined following exposure to either SF or hindlimb suspension (HLS), and during recovery from these conditions.

Methods

Subjects

Eighteen Sprague-Dawley rats (130 to 155 g) were obtained from Harlan Laboratories for the SF study. For the HLS study 20 Sprague-Dawley rats (230 to 255 g) were obtained from Simonsen Laboratories. Rats were randomly assigned to groups and were maintained on 12:12 hr light:dark cycle with food and water available *ad libitum* throughout the experiments.

Procedures

SF Conditions. During the 14 day flight on STS-58, rats (n=6) in the Flight (FL) condition were maintained individually in

cages measuring 10.2 X 10.2 X 20.3 cm. Rats (n=6) in the Flight Control (FC) condition were housed in cages of the same size in the ground colony room. Vivarium Control (VC) rats (n=6) were housed in standard rat colony cages modified by placing a clear acrylic divider lengthwise to create two sections measuring 22.9 X 45.7 X 20.3 cm. VC rats could rear during the 14 days of the flight while those in the FC and FL conditions could not. Animals in the VC and FC conditions were placed into appropriate cages on the first day following launch. All animals were transferred from the cages used during the flight period into metabolic cages (30.5 X 30.5 X 30.5 cm) between 6 and 7 hr after the time of landing (i.e., immediately after the initial test).

HLS Conditions. HLS was accomplished with a modified version of the Morey-Holton technique [4]. A device was constructed using Fas-Trac to attach a connector to the rat's tail. This connector then was attached to a swivel hook that allowed free, 360° rotation within the 30.5 X 30.5 X 30.5 cm cage. The height of this hook was adjusted so the hindlimbs of the rats were just off the floor when in full extension. With this procedure the hindlimbs of the rats (n=10) were "unloaded" (HLU) from postural (anti-gravity) support, and the animals moved about by propelling themselves with the forelimbs. Rats (n=10) in the control group (HLC) lived in similar cages but were not attached to the suspension system.

Testing Procedures. Beginning 6 hr after landing SF rats were encouraged to locomote across a walkway (15 X 30 X 150 cm) with clear acrylic walls and a glass floor. Light was passed through the glass from the front to the back edge so that foot contact could be viewed from below and recorded on videotape [5]. This video record was combined with a profile view of the rats on a split-screen display. Following the initial test on the day of landing each rat was tested after 2, 4, 7 and 14 days of recovery (Days R2, R4, etc.). Quantitative assessments of posture and limb movements were made by determining X-Y coordinates of identifiable points using a PEAK™ Technologies Motion Analysis system.

In the HLS study rats were tested within 5 min following removal from the suspension device (R0) and then on R2, R7, and R14. (In addition, these animals were exposed to 3-5 tests of the air-righting reflex and a 45-s swim test prior to testing for gait on each test day.)

Results

Posture

When first tested 6 hr. after return from SF (R0) FL rats walked slowly with the back dorsiflexed, the hindquarters lower than in FC and VC animals and with the tail dragging on the floor. Limb movements of FL rats could be described as "hesitant." Immediately after removal from HLS the rats also walked slowly, but HLU rats walked with the back straight or ventro-flexed, the hindquarters higher than HLC rats and with the tail held off the floor. Both FL and HLU rats walked with a sinusoidal, vertical oscillation of the pelvic region.

Foot Placement and Hindlimb Extension during Walking

On R0 SF rats walked with extreme dorsiflexion of the ankle (plantar extension) producing atypical foot placement that resembled that seen in 10-day old rats [6] in which the foot pads contact the floor only at the end of the stance phase. In contrast, all HLS rats walked with normal foot-pad contact.

The elevation of the hindquarters and extension of the hindlimb observed in the assessment of posture were examined further by evaluating the distances from the base of the tail to the floor (Fig. 1A) and to the foot (Fig. 1B) respectively during walking. The base of the tail was significantly closer to the floor on R0 in FL ($p < .01$) than in FC or VC rats. On R2 the base of the tail of FL rats was higher than in FC or VC rats ($p < .01$), but was not different on R7 or R14. In contrast, the base of the tail was significantly higher off the floor in HLU animals than in control rats on R0 through R7 ($ps < .001$), but not different from control rats on R14. The hindlimbs were more flexed in FL than in FC or VC rats on R0 ($p < .005$) but more extended on R2 through R7 ($ps < .05$). The hindlimb was more extended in HLU rats than in control rats on R0 through R7 ($ps < .03$), but not different from control rats on R14.

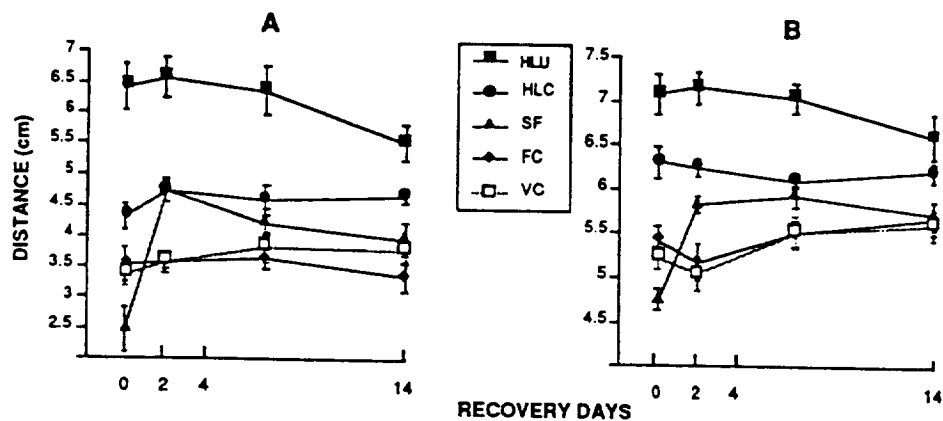


Figure 1. Elevation of the hindquarters measured as distance from the base of the tail to the surface of the walkway (Panel A) and leg extension measures as distance from the base of the tail to the foot (Panel B)

Discussion

The hindlimb extension, dorsiflexion of the ankle, and vertical oscillation of the pelvic region observed in FL and HLU rats may result from an altered balance of flexor-extensor muscles that is produced by treatments which decrease the "mechanical use" of weight-bearing muscles. Atrophic effects in SF and HLS rats are muscle-specific with slow extensors most affected, fast extensors moderately affected and flexors least affected [7, 8]. The effects of SF on physiological properties

of muscle are less well known, but documented protein changes in muscle are associated with lower excitability of the extensor pool while the flexor pool is unaffected. Assessments of muscle function in Salyut crewmembers indicated decreased strength and an increased ratio between maximum amplitude of EMG and muscle torque in leg extensors with no change in flexors [9]. These changes presumably result in a shift toward relative dominance of flexors over extensors.

Flexor dominance could produce dorsiflexion of the ankle during stationary stance as reported here. In addition, when there is extreme atrophy of the soleus, the relatively less compromised biarticular gastrocnemius may become increasingly important in dynamic ankle extension. Compromised activity of the soleus could contribute to poor adjustment of the foot prior to touchdown and to dorsiflexion during early stance when activity of fast extensors normally is minimal. Because maximal force of the gastrocnemius is length-dependent, gastrocnemius activity that contributes to ankle extension may vary as the length of the muscle changes due to biomechanical factors related to knee and ankle extension. Such changes in force could produce the vertical oscillation of the pelvis observed here. Hyper-extension of the leg in SF and HLU rats may be a postural adjustment to facilitate ankle movement by adjusting gastrocnemius length to produce proper force for adaptive ankle extension.

Footnotes

Both experiments conformed to the Center's requirements for the care and use of animals. Support was provided by NASA Grant NCC 2-723 and SJSU Foundation Grant 34-1614-0071 to R.A. Fox.

References

1. Parker, DE, Reschke, MR, Ouyang, L, Arrott, AP, et al., *Aviat Space Environ Med* 1985; 56: 601-606.
2. Dietz, V, *Physiol Rev* 1992; 72: 33-69.
3. Gazenko, OG, Grigorian, RA, Kreidich, YuV, Aizikov, GS, et al. In: Flohr, H, ed. *Post-Lesion Neural Plasticity*. Berlin: Springer-Verlag, 1988;331-343.
4. Wronski, TJ, Holton, EM, *Aviat Space Environ Med* 1987; 58: 63-68.
5. Clarke, KA, *Behav Res Meth Instr Computing* 1992; 24: 407-411.
6. Clarke, KA, Williams, E, *Physiol Behav* 1994; 55: 151-155.
7. Jiang, B, Roy, RR, Polyakov, IV, Krasnov, IB, Edgerton, VR *J Appl Physiol* 1992; 73: 107S-111S.
8. Roy, RR, Baldwin, KM, Edgerton, VR, *Exer Sports Sci Rev* 1991; 19: 269-312.
9. Kozlovskaya, IB, Aslsnova, IP, Grigorieva, LS, Kreidich, YuV, *The Physiologist* 1982; 25: S49-S52.

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SUPPRESSION OF OTOLITH-SPINAL REFLEX BY CHRONIC HYPERGRAVITY EXPOSURE AND STREPTOMYCIN TREATMENT. N. Daunton¹, R. Fox², M. Corcoran¹, P. Taber² and L. Wu². Gravitational Research Branch, NASA Ames Research Center, Moffett Field, CA and San Jose State University, San Jose, CA.

Recent behavioral studies have shown that chronic hyperG exposure disrupts air-righting, swimming, and orientation of rats. Similar effects are seen in animals chronically treated with the ototoxic drug, Streptomycin (STP), as well as in mice and rats with congenital otoconial deficiencies. To determine whether these behavioral disruptions following hyperG or STP exposure could be due to a decrease in gain in the otolith-spinal system, the otolith-spinal reflex (OSR) in response to sudden free-fall (40 cm, rat in prone position) was monitored. Following either 14 days of hyperG (2G or 3G) or 35 days of STP treatment (400 mg/kg, i.m.), pairs of EMG recording electrodes were placed in the lateral gastrocnemius and tibialis anterior muscles of the left hindlimb of each rat, under brief isoflurane anesthesia. When the animal recovered from anesthesia and had normal EMG activity, at least 15 trials of OSR were collected. Each trial was evaluated for latency and amplitude of the response. Rats exposed to either hyperG (n=9) or STP (n=7) showed significant attenuations of the OSR as compared with control animals (for hyperG n= 6; for STP n=4). Following these treatments, the response was absent or extremely low in amplitude (<2.25 mv) on over 61% of trials, depending on the combination of muscles studied and treatment used, while in controls low-amplitude responses were found in less than 26% of trials. These results suggest that gain in the otolithic portion of the vestibular system may be decreased following chronic hyperG exposure as well as after chemical destruction of otolithic hair cells. (Supported by NASA: Task 199-16-12-01 and Cooperative Agreement NCC2-723)

TIME-COURSE OF BEHAVIORAL ADAPTATION TO HYPER-GRAVITY

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Introduction

As part of an effort to determine neural mechanisms underlying sensory-motor adaptation to altered gravitational environments several functional behaviors, sensitive to changes in gravity, were identified. Our objective was to select behaviors useful for studying specific neural mechanisms by identifying behaviors that indicate specific neural systems and reflect the time-course of changes in those systems.

Because it is not practical to test animals during exposure to hyper-gravity, our approach was to study readaptation to normal gravity (1G) following adaptation to hyper-gravity. Thus, animals were tested immediately after chronic exposure to hyper-gravity. Exposure to 2 times normal gravity (resultant = 2G) was produced by centrifugation. The extent of adaptation during exposure to 2G was inferred from results of tests conducted immediately after removing animals from the centrifuge. Additional tests were conducted on some animals after 2 or 8 days of re-adaptation to normal gravity.

Immediately following centrifugation ranging from 1 to 16 days, animals were tested to assess the degree of adaptation to 2G. Tests included procedures to assess vestibular function (air-righting and orientation during swimming) and locomotion (analysis of limb movements during beam walking and swimming).

Methods

Subjects. Male, Sprague Dawley rats weighing 200 to 220 g were used. The rats were housed in standard shoebox cages and were maintained on a 12:12 hr light:dark cycle with food and water available *ad libitum* throughout the experiment.

Centrifugation. Rotation and 2G treatments were produced on a centrifuge rotated at 20.5 rpm (123°/s). Rats exposed to 2G were placed in gimbal cages at a 12-ft. radius, while animals exposed to rotation (resultant G-level = 1.03G) were in similar cages at 1.7-ft. radius. Rats in non-rotation Control Groups were housed off the centrifuge, but in the same room.

Air Righting Reflex. The righting reflex was used to assess changes in vestibular function and neuromuscular coordination. Each rat was dropped 45 cm from a supine position into a tank of warm water. The release, body segment righting (e.g., head, torso, hindlimbs), and landing attitude of each rat were monitored by video recording (Pellis, Pellis, & Teitelbaum, 1991). In conjunction with the righting reflex test, video recordings of the animals as they resurfaced after the drop were made to assess ability to orient correctly with respect to gravity.

Locomotion. Animals swam for 45 s in conjunction with the righting reflex test. Leg movements were characterized and time swimming underwater was determined from video records.

Animals walked on a beam (2.5 cm wide and 1 m long) placed inside an clear acrylic tank filled to a depth of 5 cm with ice water. One end of the beam was covered with water, while the other end (goal) was elevated (13° incline). The elevated end of the beam could be lowered to place most of the beam under water, thus encouraging hesitant animals to walk. Gait was analyzed from video records.

Results

Vestibular Function and Orientation. Significant deficits occurred in the righting response after exposure to 2G. Deficits were apparent in animals exposed to 2G for 4 days (17% righting, $p < .001$) and for 16 days (17% righting; $p < .0002$). The righting response of animals in the Rotation Groups (100% and 83%) did not differ from that of animals in the Control Groups (87% and 92% righting, $p > .30$ for all groups).

Recovery of the righting

reflex during readaptation to 1G was assessed in animals exposed to 2G for 16 days. Recovery did not occur in 2 days, but by 8 days after removal from the centrifuge the righting response of 2G animals was not significantly impaired (see Fig. 1).

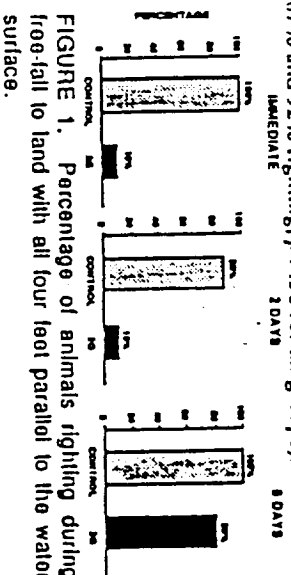


FIGURE 1. Percentage of animals righting during free-fall to land with all four feet parallel to the water surface.

Animals exposed to 2G for 4 days showed significant increases (63%) in the time taken to resurface. Some animals swam in a downward, rather than upward direction, and some were observed to dive under the water after initially reaching the surface. The increase in time to resurface (31%) seen in animals exposed to 2G for 16 days was not different from Controls. Rotation animals did not differ significantly from Control animals in resurfacing ability, suggesting that the effect of centrifugation on orientation is due to the hyper-G component rather than the angular velocity component of centrifugation.

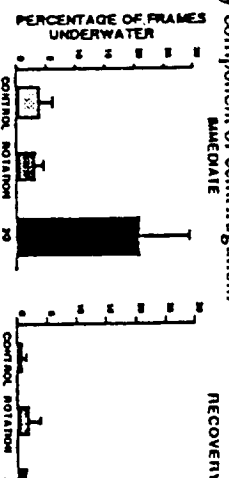


FIGURE 2. Percentage of frames rats swam underwater to 1G there was no immediately following exposure to 2G and after 2 days of difference between 2G and recovery in 1G.

Control animals in the percentage of time spent underwater (see Fig. 2).

The effect of centrifugation on time spent underwater was investigated further in a second experiment by examining rats after exposures of 1, 2, 3, or 4 days of centrifugation. Rats exposed to 2G swam underwater a greater proportion of the time (20.5%) than either the Rotation (1.8%) or Control (4.6%) animals ($p < .001$), but duration of exposure to 2G had no effect ($p > .05$). Thus, the effects of 2G on this response are produced and dissipate (or are reversed) quickly. Because there is no effect on rats exposed to rotation only, these effects appear to be due to 2G, not to rotation.

Analysis of leg movements during swimming revealed increased recruitment of the forelimbs in animals exposed to 2G. Rats in the Control and Rotation Groups held their forelimbs close to the body and seldom moved them while swimming in the open tank away from the walls. Rats exposed to 2G often moved their forelimbs in a manner resembling a "dog paddle" while swimming in the open tank. No changes or disruptions of hindlimb movements were identified.

Locomotion. Analysis of the effects of adaptation to 2G on gait was conducted by examining the sequencing of foot movements and the relationship between stance duration and speed of locomotion.

Sequencing of foot movements (stride sequences) was assessed by determining the percentage of steps that occurred in the typical lateral sequence, diagonal complement pattern. This typical pattern was disrupted significantly after 4 days of exposure to 2G; disruption was greater after 16 days.

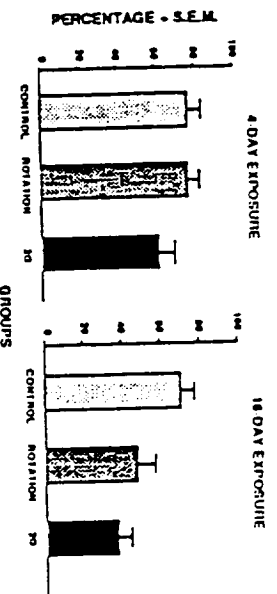
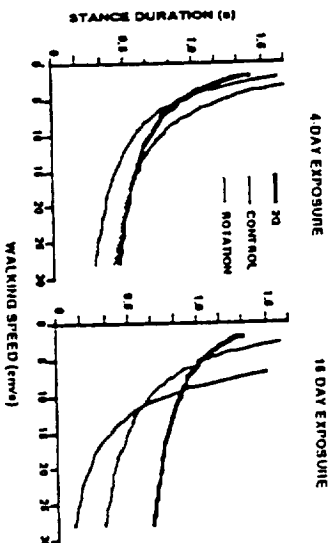


FIGURE 3. Percentage of normal sliding sequences after 4 and 16 days of exposure to 2G.

was disrupted in Rotation animals after 16 days of exposure, but not after 4 days (see Fig. 3).

The relationship between stance duration and speed of locomotion was assessed using the method of Ilriska, Kennedy, and Silberfeld (1979). Data, reported only for the hind feet (see Fig. 4), were fitted using the equation $\log Y = a + b(\log X)$. Although the rats traversed the beam at a slow walk, the general relationship between stance duration and speed of locomotion was maintained in animals in the Control and Rotation Groups, and in animals exposed to 2G for 4 days. However, after exposure to 2G for 16 days stance time was lengthened, particularly at higher speeds.



Discussion

Maximal effects on the behaviors identified in these studies occur following different durations of exposure to altered gravity. This finding suggests that adaptation of the various neural systems underlying these behaviors occurs at different rates. These effects could be produced by adaptive changes in sensory systems, neuromuscular systems, or in central motor programs.

Preliminary results indicate some effects are related to changes in vestibular sensory function. Rats exposed to 2G appeared disoriented during resurfacing, showed deficits in the righting reflex, and swam underwater more than Controls. Changes in swimming patterns also have been documented in chichlid fish and clawed toads following exposure to hyper-gravity (Rahmann, Slenczka, & Hiltig, 1990). Evidence for changes in sensory function comes from two sources. The number of synapses on Type II hair cells in the utricular macula of rats is reduced by exposure to 2G (Dauntun et al., 1991). Ultrastructural changes also occur in eichlid fish exposed to hyper-gravity during early development (Slenczka, et al., 1990). Morphological changes that lead to reduction in

sensory function could produce reduced vestibular sensitivity to gravity and result in disorientation with return to the 1G environment.

Disruption of the typical relationship between stance duration and speed of locomotion may indicate the effects of changes in neuromuscular functions. Leg movements during swimming, where proprioceptive influence is reduced, were not disrupted, perhaps indicating that central motor patterns were minimally affected during adaptation to 2G. The disruption of the timing of leg movements may indicate changes in local neuromuscular networks. Such changes could occur due to alterations in proprioceptive feedback that coordinates timing of leg movements (Gruner & Altman, 1980) and/or in spinal mechanisms that translate central motor programs into muscular action.

References

- Dauntun, N. G., Ross, M. D., Fox, R. A., Corcoran, M. L., Cutler, L. D., & Wu, L. C. (1991). Effects of chronic hyper-gravity on the righting reflex and vestibular endorgan in the rat. *Neuroscience Abstracts*, 17(1), 316.
- Gruner, J. A. & Altman, J. (1980). Swimming in the rat: Analysis of locomotor performance in comparison to stepping. *Exp. Brain Res.*, 40, 374-382.
- Ilriska, R. E., Kennedy, S., & Silberfeld, E. K. (1979). Quantitative aspects of normal locomotion in rats. *Life Science*, 25, 171-180.
- Pellis, S. M., Pellis, V. C., & Teitelbaum, P. (1991). Air righting without the cervical righting reflex in adult rats. *Behav. Brain Res.*, 45, 185-188.
- Rahmann, H., Slenczka, K., & Hiltig, R. (1990). Effect of hyper-gravity on the swimming behavior of aquatic vertebrates. *Proceedings of the Fourth European Symposium on Life Sciences Research in Space*, ESA SP-307, 259-263.
- Slenczka, K., Anken, R., Bauerle, A., Kortje, K. H., Paulus, U., & Rahman, H. (1990). Electromicroscopical and biochemical aspects of hyper-gravity conditions during early ontogenetic development of chichlid fish. *Proceedings of the Fourth European Symposium on Life Sciences Research in Space*, ESA SP-307, 321-328.

63.13

TAIL SUSPENSION WITH AND WITHOUT HINDLIMB UNLOADING AFFECTS NEUROMUSCULAR FUNCTION IN THE ADULT RAT. R.A. Fox¹, N.G. Daunton², M.L. Corcoran², L.C. Wu¹ and F. D'Amelio¹, ¹San Jose State Univ., San Jose, CA 95192; ²NASA Ames Res. Ctr, Moffett Field, CA 94035. Tail suspension simulates some of the effects of spaceflight by unloading the hindlimbs and producing changes in the structure, physiology and biochemistry of the hindlimb neuromuscular system. We reported effects of suspension on swimming (Corcoran et al., 1990), but effects on other neuromuscular functions of adult rats have not been documented, nor have the effects of tail restriction (a consequence of suspension) without unloading. Thus, neuromuscular coordination was assessed following 14 days of hindlimb suspension (S) and of restricted tail usage (TR) in 10 rats per group. Animals were tested immediately after suspension, and again after 2, 7, 14, 21 & 28 days. Various aspects of righting, swimming and walking were disrupted in both S and TR animals as compared with Controls. In S animals, air righting was significantly delayed, speed of swimming and walking were significantly slowed, and the hindlimbs hyper-extended during walking. Also, an increase in hindlimb stride width and a shift toward moving only one foot at a time were seen, indicating an apparent attempt to enhance stability during walking. In both S and TR animals the forelimbs were used during swimming, a response normally seen only in neonatal rats, and animals swam at a significantly steeper angle. In addition, during walking, the forelimbs of S and TR animals were placed wider apart and the pelvis was elevated. Recovery rates varied, but some effects lasted at least 21 days, demonstrating that both hindlimb suspension (unloading) and restricted tail usage can produce long-lasting effects on neuromuscular function in adult rats.



STUDY OF ADAPTATION TO ALTERED GRAVITY THROUGH SYSTEMS ANALYSIS OF MOTOR CONTROL

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ABSTRACT

Maintenance of posture and production of functional, coordinated movement demand integration of sensory feedback with spinal and supra-spinal circuitry to produce adaptive motor control in altered gravity (G). To investigate neuroplastic processes leading to optimal performance in altered G we have studied motor control in adult rats using a battery of motor function tests following chronic exposure to various treatments (hyper-G, hindlimb suspension, chemical destruction of hair cells, space flight). These treatments differentially affect muscle fibers, vestibular receptors, and behavioral compensations and, in consequence, differentially disrupt air righting, swimming, posture and gait. The time-course of recovery from these disruptions varies depending on the function tested and the duration and type of treatment. These studies, with others (e.g., D'Amelio *et al.* in this volume), indicate that adaptation to altered gravity involves alterations in multiple sensory-motor systems that change at different rates. We propose that the use of parallel studies under different altered G conditions will most efficiently lead to an understanding of the modifications in central (neural) and peripheral (sensory and neuromuscular) systems that underlie sensory-motor adaptation in active, intact individuals.

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INTRODUCTION

While it is well known that most individuals learn to operate effectively in an altered G environment after living for a few days in that condition, little is known about the neural mechanisms that underlie this adaptation in sensory-motor systems. The main thrust of our research over the past few years has been to begin to identify those neural mechanisms so that eventually we will be able to answer the question: What neural changes occur that allow the individual to operate efficiently and effectively after a few days in new gravitational environments? Our approach to answering this question has involved the development in rats of behavioral measures that reflect the extent and time course of changes in postural reflexes, orientation and locomotion induced by chronic exposure to hyper-G. We have used the results of these behavioral studies to identify specific locations to study in the CNS and to determine the timing of our search for modifications (e.g., structural, neurochemical, electrophysiological) in the nervous system that underlie those changes in sensory-motor control.

Altered G Studies

When an animal that has developed and lived in the normal 1G environment of Earth is exposed to a different gravitational environment, adjustments at all levels of the posture, orientation, and movement control systems must take place if the individual is to operate efficiently and effectively in that new environment. A growing body of information is available on sensory-motor adaptation during and following exposure to micro-G. Changes have been reported in motor performance, spatial orientation, postural control, equilibrium, and gaze control. In addition, perceptual problems and motion sickness often occur (Daunton, 1996). Of specific importance to this work are the findings of changes in morphology in otolithic endorgans and in physiology of otolith-spinal systems during and following micro-G exposure. In morphological studies Ross (1993) has shown that the number of synapses in

utricular hair cells increases after exposure to microgravity, while the opposite effect is found following hyper-G exposure. Electrophysiological data obtained during and following flight have shown changes in both spontaneous and evoked activity in otolithic afferents and otolith-related units in vestibular nuclei (see Daunton, 1996), while evidence for alterations in otolith-mediated vestibulo-spinal reflexes during and following spaceflight has also been shown (Watt *et al.*, 1986; Reschke *et al.*, 1986; see Daunton, 1996).

Because of the difficulties involved in testing animals on the centrifuge during chronic hyper-G exposure, there is little information on sensory-motor adaptation during exposure to hyper-G. Rather, the bulk of data comes from studies conducted at 1G immediately following chronic exposures to 2G, and during re-adaptation to normal 1G (i.e., recovery). Evidence for modification of behaviors (e.g., posture, locomotion, swimming, righting, nystagmus) related to vestibulospinal and vestibulo-ocular function following hyper-G exposure has been reported (e.g., Smith, 1975; Fox *et al.*, 1992, 1993; Clark, 1974, 1976; Rahmann *et al.*, 1990). In studies of the neural substrate following hyper-G exposure, modifications in brainstem, cerebellum, and sensory-motor cortex have been shown (e.g., Grenell *et al.*, 1968; Johnson *et al.*, 1976; Krasnov *et al.*, 1986; Slenzka *et al.*, 1990).

Sensory-motor Adaptation

As employed in this paper, the term "adaptation" implies a goal-directed process that tends to readjust control systems to new environmental constraints, so that optimal functioning of the organism is attained. In altered G, adjustments must be made to the changed relationships between sensory inputs and motor outputs generated during active movement in the new gravitational conditions. Those alterations are due in part to the effects of abnormal gravity on the vestibular system, since a portion of this system has evolved specifically to sense gravitational as well as other types of linear acceleration. Thus, this system is particularly affected by alterations in the gravitational environment. Altered vestibular input leads to disruption of the normal relationships between sensory inputs and motor outputs, causing problems in control of posture, orientation, and movement. Sensory-motor adaptation, however, results in the optimization of control after a period of time in altered G.

The adaptation process is thought to be initiated by an "error" or mismatch between the actual and expected sensory inputs generated by active movement (behavior) in the altered conditions. The "error" or mismatch is minimized over time as adaptation occurs and sensory-motor control is re-optimized for the new conditions (Melvill Jones, 1983). Adaptation in these systems thus involves a re-learning of sensory-motor relationships based on the results of motor responses in the new or altered environment. It should be emphasized that active, voluntary movement appears to be necessary for adaptation to occur. For example, much as one can learn to ride a bicycle only by actively balancing and making appropriate leg movements, but not by simply observing, or passively riding as a passenger, effective adaptation to new conditions requires active participation.

The neural processes involved in sensory-motor adaptation are thought to be similar to those involved in learning and/or in recovery from neural damage, i.e., experience-dependent neural plasticity (Lüneburg and Flohr, 1988; Melvill Jones and Berthoz, 1985; Singer, 1989). It is likely that similar generic types of changes in the CNS (e.g., neuromodulation, changes in synaptic efficacy, "rewiring", synaptic sprouting, reorganization of neural networks) underlie sensory-motor adaptation to various eliciting conditions (e.g., hyper-G, micro-G, prism-altered vision, loss of vestibular input). For this reason the insights obtained from studies of one model of sensory-motor adaptation should be valuable for understanding the neural substrate of adaptation in a different situation, even though the exact neural circuits and the timing of changes may not be the same in the two situations. We feel that additional insights into the underlying mechanisms of adaptation can be obtained by evaluating sensory-motor adaptation in a variety of eliciting conditions. Thus we have conducted parallel studies of control of posture, orientation and locomotion following exposure to hyper-G, micro-G, simulated micro-G (hindlimb suspension - HLS), and in some cases destruction of vestibular hair cells (streptomycin treatment) to further our understanding of the mechanisms underlying sensory-motor adaptation. We have compared the results of these parallel studies using similar paradigms and measures (behavioral - air-righting, swimming, locomotion, posture; physiological - EMG of Free-Fall Response; neurochemical - cholinergic receptors, GABA immunoreactivity, neuropeptides; and morphological - neuromuscular junctions, dorsal root ganglia, spinal cord, synapses, glia) following the different kinds of treatments.

METHODS

In this paper we shall report on results from studies in which G was altered. The effects of altered G exposure on air-righting, orientation during swimming, and EMG during Free-Fall were determined in young, male Sprague-Dawley rats. The paper by D'Amelio *et al.* (this volume) describes results of some parallel morphological and neurochemical studies conducted using the same paradigms of altered G exposure.

In all studies we have used chronic exposure to altered conditions and have permitted active movement of the animals during this exposure. Because of the technical complications of testing during chronic exposure, especially during centrifugation or space flight, the basic paradigm we have used is one in which changes induced by chronic exposure are studied in tests conducted after the exposure (e.g., after return to 1G following a period of exposure to hyper-G). Disruption in a measure in an initial test conducted soon after removal from the chronic treatment provides an estimate of the magnitude of change that occurred during the exposure, and thus estimates the degree of adaptation that has occurred. Additional tests at varying durations after removal from the chronic treatment provide an estimate of the rate of change in the systems that are readapting to normal conditions.

In our studies rats are exposed to altered conditions for varying durations (typically 14 days) following which behavioral tests (Fox *et al.*, 1992) and neurochemical and morphological assessments (D'Amelio *et al.*, 1996) are made after varying periods of recovery (usually 14 days, with tests immediately after reintroduction to the normal environment and then every few days during this recovery period). This approach is based on the concept that three stages are involved in both the adaptation to a new environmental condition and readaptation to the original gravitational condition. These stages of adaptation and readaptation involve (1) an initiation stage during which the altered conditions produce disruptions of behavior and trigger adaptive modifications, (2) a consolidation stage during which there is partial recovery of function as adaptive changes occur, and (3) a maintenance stage during which behavior is stabilized and the adaptive modifications are actively maintained (Daunton, 1996). Behavioral testing allows us to evaluate the timing of these stages for each sensory-motor system and then to relate the stage of readaptation to morphological and neurochemical changes found in the nervous system.

All of our studies have been conducted in young adult male rats (220-280 g). In studies involving centrifugation, the 24-ft Diameter Centrifuge at Ames Research Center was used. To generate a 2G environment, the centrifuge was rotated at 20.5 rpm. Animals were housed two per "shoe-box"-sized cage, with four such cages housed in a gimbaled enclosure. Rotation Control groups of animals were run in each centrifuge study to control for the effects of angular velocity. The method of Morey-Holton was used in HLS studies, with a control group used to assess the effects of immobilization of the tail. The micro-G study was conducted on animals kindly provided by E. Holton.

RESULTS

Behavioral Studies

Two behavioral responses that show significant disruption following hyper-G exposure are the righting reflex and orientation during swimming. Both of these responses are thought to be based primarily on vestibular inputs, in large part from the otolithic organs (Trune and Lim, 1983; Money and Scott, 1962)

Air-righting Reflex. The air-righting reflex (Hård and Larsson, 1975; Huygen *et al.*, 1986; Igarashi and Guittierrez, 1983; Pellis *et al.*, 1989) was tested by dropping each rat 45 cm from a supine position into a tank of warm water. The percentage of animals righting and time taken to right for each animal were evaluated using frame-by-frame analysis of video recordings.

The righting reflex was disrupted (time to right significantly increased; decrease in percentage of animals righting prior to reaching the water) in 2G but not Rotation Control animals immediately after return to 1G (Daunton, *et al.*, 1991a, b). Normal righting returned between the 5th and 8th post-centrifuge day following 14-day exposures (Fox *et al.*, 1992 see Figure 1). In animals exposed to 14 days of HLS, there is an initial deficit in righting, but normal righting recovers more rapidly, than following 2G exposure (Fox *et al.*, 1993).

The disruption of the air-righting reflex seen in animals exposed to hyper-G suggests that the threshold for detecting vertical linear acceleration (i.e., gravity) may be increased following hyper-G exposure, and for some days thereafter, an effect possibly related to the finding of decreased synapses in Type II hair cells in utricular maculae of 2G adapted animals (Daunton *et al.*, 1991b; Ross, 1993).

Swimming. Swimming was used to evaluate the ability of animals to maintain normal orientation (nearly parallel to the water surface, with nose above the water) while moving through the water - a condition in which tactile cues to orientation are reduced. Animals with labyrinthine deficiencies, particularly otoconial, swim underwater a great deal, while normal animals seldom do (Trune and Lim, 1983; Petrosini, 1984; Meza *et al.*, 1996). Video records of the animals as they swam were analysed for time spent underwater and swimming position. A comparison of the 2G vs Rotation Control and Off-Centrifuge Control animals showed that the 2G animals spent significantly more time underwater than animals in the other two groups. In many cases the animals appeared to be disoriented and to swim purposefully in a downward direction. Interestingly, this response recovered more rapidly (between 4 and 24 hrs following 2G exposure) than the air-righting response. The fact that underwater swimming was not seen following 14 days of HLS where no vestibular effects are known provides additional support for the suggestion that this abnormal response is vestibular in nature. (Daunton *et al.*, 1991a; Fox *et al.*, 1992; 1993).

Rate of Adaptation. We have looked in detail at rate of readaptation to normal G following exposures to 2G for up to 14 days as reflected by the two measures of vestibular function - air-righting and underwater swimming (Daunton *et al.*, 1995). As noted above, we have found that the righting reflex has a longer readaptation time constant (TC) than does spatial orientation during swimming. The TC for air righting is 4 days, with 50% of animals righting normally on the 4th day following 14 days of 2G exposure. For underwater swimming, the TC is around 18 hours (Daunton, unpublished data). Thus, while both of these behaviors are thought to be highly dependent on vestibular, particularly otolith, function, it appears that different neural processing is involved. During adaptation to altered G, therefore, different functional behaviors adapt at different rates, and reflect separate neural mechanisms even though they may be based on the same altered vestibular inputs.

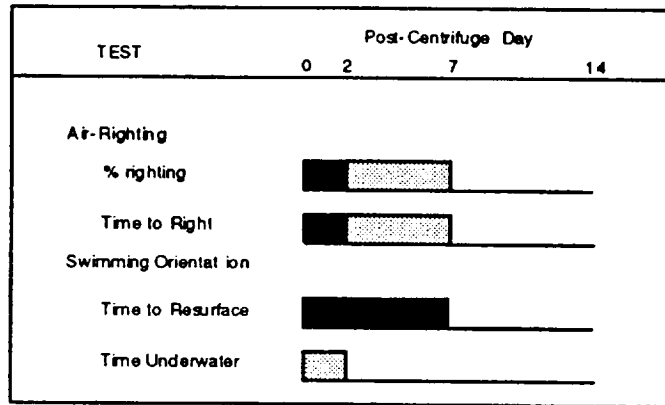


Fig. 1. Rate of readaptation after 14 days of exposure to 2 G. The initiation stage (during which no improvement in disruption occurs) is shown by filled bars. The consolidation stage (during which the response improves toward control values) is shown by stippled areas. Complete readaptation (Hyper-G animals not different from Controls) is indicated by a thin line.

Other Behavioral Measures. We have looked extensively at various aspects of gait during walking on a hard surface and at limb movements during swimming. Briefly, we have found that gait, as assessed by measures of interlimb coordination, stability adjustments, and kinematics of limb movements, is altered following 2G, HLS, and micro-G exposures. The effects last several weeks in the HLS animals, while in 2G and micro-G animals recovery is faster. Various characteristics of swimming in addition to orientation have been evaluated following HLS and 2G exposures. Following HLS, but not 2G, animals showed disrupted interlimb coordination. Following both HLS and 2G, forelimbs were recruited during swimming (as seen early in development); suppression of forelimb use returned more rapidly following 2G exposure (within ~ 2 days) than following HLS (recovery time ~ 1 week). In addition, following 2G exposure, animals swam at a flatter angle to the water surface than controls, while following HLS animals swam at a steeper angle (Fox *et al.*, 1992; 1993; 1994).

Neurophysiological Measures. The disruption of behaviors related to vestibular function (air righting and swimming) and the decrease reported in the number of synapses in the otolith end organs following exposure to hyper-G both suggest that the gain in the otolithic portion of the vestibular system may be reduced by chronic exposure to hyper-G. To assess whether there might be a decrease in gain of the vestibular input to the otolith-spinal system we have investigated the effects of chronic exposure to hyper-G on the early EMG response in hind limb muscles to a sudden drop from the prone position (Free-Fall Response - FFR). This response is dependent on the vestibular system in humans (Greenwood and Hopkins, 1976), cats (Watt, 1976) and baboons (Lacour *et al.*, 1978, 1979). Gruner (1989) has demonstrated that this response also occurs in the rat.

In this preliminary experiment animals were exposed to 2.86 G for 7 or 12 days. After removal from hyper-G, the EMG in the lateral gastrocnemius muscle of the left hind leg was recorded during 30 cm of free-fall. At least 15 trials of the FFR were collected from each animal. The FFR in control rats not exposed to centrifugation resulted in an EMG with large amplitude and consistent latency. The FFR in rats exposed to centrifugation was inconsistently elicited and thus the amplitude appears greatly suppressed (see Figure 2). These results suggest that the gain in the otolithic portion of the vestibular system may be decreased following chronic exposure to hyper-G.

DISCUSSION

The reduced magnitude of the FFR found following chronic exposure to hyper-G suggests that this treatment may produce a reduction in gain of the gravity-sensitive portion of the vestibular system. Such a reduction would be consistent with the morphological results reflecting a reduction in numbers of synapses in type II hair cells in rat utricular maculae following exposure to hyper-G (Daunton *et al.*, 1991a; Ross, 1993), and with the behavioral results indicating disruptions of air-righting and swimming orientation reviewed here. These results also illustrate a possible relationship between effects on morphology, behavior and neurophysiology uncovered by studying vestibular end organs, air righting and swimming, and FFR in parallel experiments.

Disruption of this early EMG response to simulated free-fall in astronauts during orbital flight (Watt *et al.*, 1986) also suggests a reduction in vestibular influence on the motor system. Disruption of the reflex in man and the FFR in rats both occurred when there was a change between environments that resulted in a ΔG of minus 1 (from 1G of Earth to the micro-G of spaceflight or from 2G during centrifugation to the normal 1G). While not suggesting that a ΔG from 2G to 1G is equivalent to one from 1G to 0G, it is interesting to note that the apparent reduction in magnitude in this vestibular reflex occurred following a ΔG of minus 1 in both cases. This suggests that these reductions in the force of the gravitational field may have a similar impact on this sensory-motor system. Important questions to be addressed here center around identifying the mechanism(s) and the time course of the increase in gain in the otolithic portion of the vestibular system that is produced by a positive ΔG , as, for example, by the return to the 1G environment of Earth following spaceflight. Watt *et al.* (1986) were unable to measure the rate of increase in gain to normal, pre-flight levels following return from orbit, perhaps because the change occurred too rapidly. Similarly, if one assumes that the air-righting reflex is related to gain in the otolithic

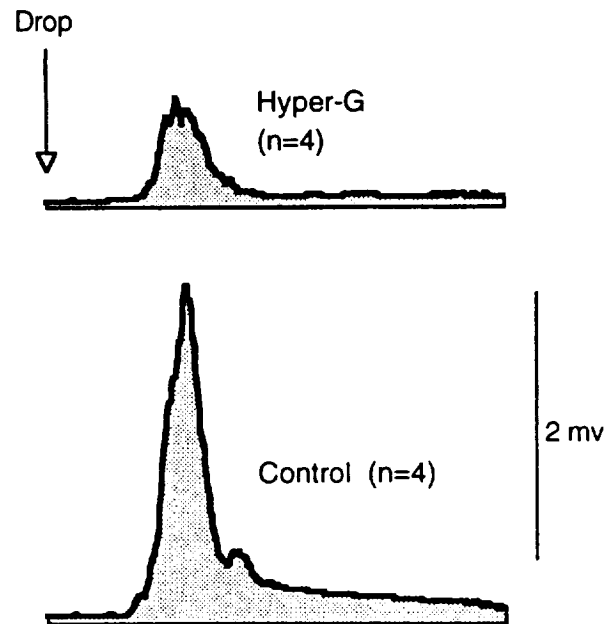


Fig. 2. Average rectified EMG of the gastrocnemius muscle. The average rectified FFR was obtained over 14 to 20 tests for each rat and then averaged to produce this figure. The moment of release is at the start of each trace indicated by the arrow labelled "drop".

portion of the vestibular system, the observation that air-righting in rats appears to be intact following spaceflight (Gazenko *et al.*, 1988) further suggests that gain may be normal or possibly increased shortly after flight.

Detailed analysis of the rate of recovery in motor responses suggests that changes underlying adaptation of whole-body actions during unrestricted, active movements may involve multiple sensory-motor systems. Underwater swimming recovers quickly after exposure to hyper-G suggesting a rapid response of the sensory-motor system with return to 1G. As measured by air-righting, however, the rate of readaptation appears considerably slower. These effects suggest that while air-righting and orientation during swimming both depend in part on vestibular input, different sensory-motor systems contribute to the two responses.

However, our studies with the ototoxic drug streptomycin (STP) suggest a more complicated picture. During chronic treatment with STP, disruption of underwater swimming develops at a slower rate than disruption of the righting reflex. This finding is the reverse of rate changes during recovery from hyper-G and suggests that more extensive damage to gravity-sensitive hair cells is required to disrupt orientation during swimming (Meza *et al.*, 1996). A significant difference in swimming between treated and control animals was found only after three weeks of treatment with STP. By that time significant underwater swimming was seen along with other abnormal swimming behavior, such as vertical barrel rolls with head down, looping, and other patterns of swimming characteristic of otoconia-deficient animals (Trune and Lim, 1983). Although head-down vertical barrel rolls have been observed in some rats adapted to 2G, these patterns occur infrequently unless animals are tested immediately after removal from hyper-G. Thus, while air-righting and motor control related to orientation during swimming both depend importantly on vestibular input under normal conditions, the fact that changes in these two measures are not coordinated in time suggests interaction with, or the involvement of additional sensory-motor systems that change at different rates.

It has become increasingly clear that the control of motor function results from complex interaction among many sensory-motor systems throughout the nervous system rather than as a collection of simple input-output relationships (Mergner and Hlavacka, 1995). Efficient motor control depends on the coordination of high order (cortical) and lower order (peripheral) components of the sensory-motor system (Georgopoulos, 1995). This interaction can include interplay between inputs from external receptors (e.g., vestibular, visual, or auditory receptors), internal sensations (e.g., proprioception and feedback from joints, tendon organs and muscle spindles) as well as models of the outside world or our subjective orientation (Lackner, 1993; Mittelstaedt, 1983). Because effective motor control in active, multi-linked organisms depends on complex interaction between various systems and forms of input, understanding of adaptation of motor control necessarily requires investigation of the entire motor system. Matthews (1995), for example, has proposed that attention to interaction among subcomponents of the sensory-motor system is critical in the study of motor control: "...the spinal cord has surrendered its autonomy to the brain, but the brain can only control the limbs by talking to the spinal cord in a language that it can understand, determined by its pre-existing circuitry; and both receive a continuous stream of feedback from the periphery." (p. vii). We feel that adaptation to a condition of altered G necessarily involves active movements and multiple sensory-motor systems. To achieve a full understanding of the mechanisms of adaptation leading to effective motor control in conditions of altered G, we suggest that parallel studies involving manipulations of both the altered G environment and the specific sensory and/or motor systems participating in motor control should be conducted.

ACKNOWLEDGMENTS

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REFERENCES

- Clark, D. L., Effects of Chronic 2g Centrifugation on Equilibrium Behavior in the Rat, *Behav. Biol.*, 12, 509 (1974).

- Clark, D. L., Effects of Weightlessness on the Development of the Vestibular Apparatus and Ocular Nystagmus in the Rat, U. S. Government Contractor Report, No. N73-17112, Ohio State University Research Foundation, Columbus, OH (1976).
- D'Amelio, F., R. A. Fox, L. C. Wu, N. G. Daunton, Quantitative Changes of GABA-Immunoreactive Cells in the Hindlimb Representation of the Rat Somatosensory Cortex after 14-Day Hindlimb Unloading by Tail Suspension, *J. Neurosci. Res.*, 44, 532 (1996).
- D'Amelio, F., R. A. Fox, L. C. Wu, N. G. Daunton, and M. L. Corcoran, Effects of Microgravity on Muscle and Cerebral Cortex: A Suggested Interaction. -- this volume -- (1996).
- Daunton, N. G., Adaptation of the Vestibular System to Microgravity, in *Handbook of Physiology: Section 4: Environmental Physiology Vol 1*, edited by M. J. Fregly and C.M. Blatteis, pp. 765-783, Oxford University Press, Oxford (1996).
- Daunton, N., M. Corcoran, R. Fox, F. D'Amelio, and I. Polyakov, Behavioral Studies on Recovery of Vestibular Function Following Chronic Exposure to Different Levels of Hypergravity, *ASGSB Bulletin*, 9(1), 66 (1995).
- Daunton, N., M. Corcoran, and M. Ross, Behavioral and Neural Adaptation to Altered Gravity, in *Research and Technology 1991*, NASA TM-103924, p. 233, NASA Ames Research Center, Moffett Field, CA (1991a).
- Daunton, N. G., M. D. Ross, R. A. Fox, M. L. Corcoran, L. Cutler, and L. C. Wu, Effects of Chronic Hypergravity on the Righting Reflex and Vestibular Endorgan Morphology in the Rat, *Neuroscience Abstracts*, 17, p. 316 (1991b).
- Fox, R. A., M. Corcoran, N. G. Daunton, and E. Morey-Holten, Effects of Spaceflight and Hindlimb Suspension on the Posture and Gait of Rats, in *Vestibular and Neural Front*, edited by K. Taguchi, M. Igarashi, and S. Mori, Elsevier Science B.V., Amsterdam (1994).
- Fox, R. A., N. Daunton, M. Corcoran, and L. Wu, Time-course of Behavioral Adaptation to Hypergravity, in *Posture and gait: Control mechanisms*, edited by M. Woolacot and F. Horak, pp. 178-181, University of Oregon Books, Eugene, OR (1992).
- Fox, R. A., N. Daunton, M. Corcoran, and L. Wu, and F. D'Amelio, Tail Suspension with and without Hindlimb Unloading Affects Neuromuscular Function in the Adult Rat, *Neurosci. Abstracts*, 19, 147 (1993).
- Gazenko, O. G., R. A. Grigorian, Yu. V. Kreidich, G. S. Aizikov, and A. S. Markin, Compensation of Cerebellar-Vestibular Lesions and Microgravity, in *Post-Lesion Neural Plasticity*, pp. 331-343, Springer-Verlag, Berlin (1988).
- Georgopoulos, A. P. Current Issues in Directional Motor Control, *Trends in Neuroscience*, 18(11), 506-510, (1995).
- Greenwood, R., and A. Hopkins, Muscle Responses During Sudden Falls in Man, *J. Physiol.*, 254, 507 (1976).
- Grenell, R. G., H. Hazama, M. Nakazawa, and E. Einberg, Effects of Gravitational Changes on RNA of Cerebral Neurons and Glia. I. RNA Changes of Deiters' Cells and Glia, *Brain Res.*, 9, 115 (1968).
- Gruner, J. A., Comparison of Vestibular and Auditory Startle Responses in the Rat and Cat, *J. Neurosci. Methods*, 27, 13 (1989).
- Hård, E., and K. Larson, Development of Air Righting in Rats, *Brain Behav. Evol.*, 11, 53 (1975).

- Huygen, P., A. Fixcher, and W. Kuijpers, The Vestibular Functions of the Manganese-Deficient Rat, *Acta Otolaryng.*, 101, 19 (1986).
- Igarashi, M., and O. Guittierrez, Analysis of Righting Reflex in Cats with Unilateral and Bilateral Labyrinthectomy, *ORL*, 45, 279 (1983).
- Johnson, J. E. J., W. R. Mehler, and J. Oyama, The Effects of Centrifugation on the Morphology of the Lateral Vestibular Nucleus in the Rat: A Light and Electron Microscopic Study, *Brain Res.*, 106, 205 (1976).
- Krasnov, I. B., L. N. D'yachkova, I. I. Babichenko, and V. N. Anders, The Ultrastructure of the Cerebellar Cortex in Rats Exposed to Altered Gravitational Force, *Space Biol. Med. Digest*, 14, 279 (1986).
- Lackner, J. R., Orientation and Movement in Unusual Force Environments, *Psychol. Sci.*, 4(3), 134 (1993).
- Lacour, M., C. Xerri, and M. Hugon, Muscle Responses and Monosynaptic Reflexes in Falling Monkey: Role of the Vestibular System, *J. Physiol.*, 74, 427 (1978).
- Lacour, M., C. Xerri, and M. Hugon, Compensation of Postural Reactions to Fall in the Vestibular Neurectomized Monkey. Role of the Remaining Labyrinthine Afferences, *Exp. Brain Res.*, 39, 363 (1979).
- Lüneburg, U., and H. Flohr, Effect of Substance P on Vestibular Compensation, in *Post-Lesion Neural Plasticity*, edited by H. Flohr, pp. 699-704, Springer-Verlag, Berlin (1988).
- Mathews, P. B. C. Forward, in *Neural Control of Movement*, edited by W. R. Ferrel, and U. Proske, p. ix, Plenum Press, New York, NY (1995).
- Melville Jones, G., Auto-adaptive control of central plasticity: Observations and Speculations, in *Synegetics of the Brain*, edited by E. Basar, H. Flohr, H. Haken, and A. J. Mandell, pp. 122-138, Springer-Verlag, Berlin (1983).
- Melville Jones, G., and A. Berthoz, Epilogue, A Compendium of Questions Concerning Adaptive Mechanisms, in *Adaptive Mechanisms in Gaze Control: Facts and Theories*, edited by A. Berthoz and G. Melville Jones, pp. 341-345, Elsevier, New York (1985).
- Mergner, T., and F. Hlavacka, Preface, in *Multisensory Control of Posture*, edited by T. Mergner, and F. Hlavacka, pp. v-vi, Plenum Press, New York, NY (1995).
- Meza, G., B. Bohne, N. Daunton, R. Fox, and J. Knox, Damage and Recovery of Otolithic Function Following Streptomycin Treatment in the Rat, in *New Directions in Vestibular Research*, *Ann. N. Y. Acad. Sci.*, 781, 666 (1996).
- Mittelstaedt, H., Subjective Vertical in Weightlessness, in *Vestibular and Visual Control on Posture and Locomotor Equilibrium*, edited by M. Igarashi and F. O. Black, pp. 139-150, Karger, Basel (1983).
- Money, K. E., and J. W. Scott, Functions of Separate Sensory Receptors of the Auditory Labyrinth of the Cat, *Am. J. Physiol.*, 202, 1211 (1962).
- Pellis, S., V. Pellis, T. Morrissey, and P. Teitelbaum, Visual Modulation of Vestibularly-Triggered Air-Righting in the Rat, *Behav. Brain Res.*, 35, 23 (1989).
- Petrosini, L., Task-Dependent Rate of Recovery from Hemilabyrinthectomy: An Analysis of Swimming and Locomotor Performances, *Physiol. Behav.*, 33(5), 799 (1984).

- Rahmann, H., K. Slenzka, R. Hilbig, Effect of Hyper-Gravity on the Swimming Behavior of Aquatic Vertebrates, in Fourth European Symposium on Life Sciences Research in Space, ESA SP-307, pp. 259-263 (1990).
- Reschke, M., D. Anderson, and J. Homick, Vestibulospinal Response Modifications as Determined with the H-Reflex During the Spacelab-1 Flight, *Exp. Brain Res.*, 64, 367 (1986).
- Ross, M. D., Morphological Changes in the Rat Vestibular System Following Weightlessness, *J. Vest. Res.*, 3, 241 (1993).
- Singer, W., Dynamics and Plasticity of Neuronal Systems: Introductory Remarks, in *Dynamics and Plasticity in Neuronal Systems*, edited by N. Elsner and W. Singer, pp. 7-8, Georg Thieme Verlag, New York, NY (1989).
- Slenzka, K., R. Anken, A. Bäuerle, K. Hl Körtje, U. Paulus, and H. Rahmann, Morphological, Electronmicroscopical and Biochemical Aspects of Hyper-Gravity Conditions During Early Ontogenetic Development, in Fourth European Symposium on Life Sciences Research in Space, ESA SP-307, pp. 321-328 (1990).
- Smith, A. H., Principles of Gravitational Biology, in *Foundations of Space Biology and Medicine*, edited by M. Calvin and O. G. Gazenko, pp. 129-162, NASA, Washington, DC (1975).
- Trune, D. R., and D. J. Lim, The Behavior and Vestibular Nuclear Morphology of Otoconia-Deficient Pallid Mutant Mice, *J. Neurogenet.*, 1(1), 53 (1983).
- Watt, D. G. D., Responses of Cats to Sudden Falls: An Otolith-Originating Reflex Assisting Landing, *J. Neurophys.*, 39(2), 257 (1976).
- Watt, D. G. D., K. E. Money, and L. M. Tomi, M.I.T./Canadian Vestibular Experiments on the Spacelab-1 Mission: 3. Effects of Prolonged Weightlessness on a Human Otolith-Spinal Reflex, *Exp. Brain Res.*, 64, 308 (1986).

173.14

FUNCTIONAL DEAFFERENTATION OF KNEE JOINT AFFERENTS PRODUCES LEG EXTENSION AND KNUCKLE WALKING IN RATS. R.A. Fox*, J. Knox, J. Skinner, and M. Spomer. Dept. of Psych., San Jose St. Univ., San José, CA 95192-0120.

The knee joint of Sprague Dawley rats (n=24) was anesthetized to investigate the role of joint afferent signals in gait. Intra-articular injections (.2 or .3 ml) of 40 mg/ml lidocaine-HCl were performed under gas anesthesia (2% isoflourane) in a repeated measures design with the experimental (injection) condition counterbalanced with two control conditions: (a) injection of saline to control for the effects of pressure in the joint and (b) gas anesthesia alone.

The effects of anesthetically "deafferenting" the knee on limb kinematics were assessed from videotapes of the animals as they (a) traversed a 1m walkway, (b) swam across 1-m long channel, and (c) trotted (at .27 m/s) on a treadmill. Tests of swimming and walking occurred 2-6 min. following injection of lidocaine and at approximately 10 min. following injection when foot placement began to become normal. Treadmill tests occurred from 8-20 min. after injection.

During anesthetic "deafferentation" of the knee joint rats walked with the toes clenched (i.e., "knuckle" walking), the ankle hyper-extended, the range of motion in the knee reduced in swing, and with extreme vertical oscillation of the hip. Ankle effects were present in swimming but toe clenching was absent. These effects suggest disruption of knee afferents may prevent normal "gating" of tactile stimulation of the foot producing extensor dominance and inhibiting flexor activity that normally initiates the swing phase of locomotion.

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804.1

EFFECTS OF SECTION AND SPONTANEOUS REATTACHMENT OF THE SOLEUS MUSCLE ON THE GAIT OF THE ADULT RAT. B. A. Fox*, C. Moreno, and J. Knox. Department of Psychology, San José State University, San José, CA 95192-0120.

Biomechanical action of the soleus muscle was eliminated by sectioning the soleus muscle at the Achilles tendon in both hind legs of six adult male Sprague-Dawley rats. Gait of the rats was studied through analysis of movements of the hind legs during 7 days of recovery. Foot placement was assessed as the rats spontaneously walked across a 1-m walkway and limb kinematics were evaluated during locomotion on a treadmill in horizontal and inclined (10 deg) positions. Limb coordination was characterized in a swim test. All tests were administered prior to surgery to determine baseline performances and then at 4, 24, and 168 hr. after surgery.

At 4 hr. after surgery the rats walked in a slightly crouched posture with the ankle dorsiflexed and with normal limb coordination. Crouching and dorsiflexion of the ankle increased at 24 hr. post surgery. Normal ankle flexion returned by 168 hr. but the previously normal limb coordination was disrupted at this time. Post mortem examination indicated the soleus muscle had attached to the lateral gastrocnemius. These results suggest that the soleus muscle is not critical for typical, coordinated hind leg movement in the adult rat. The uncoordinated leg movements observed after the soleus attached to the gastrocnemius suggest that the CNS had not adapted sufficiently to produce smoothly controlled ankle movement.

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Damage and Recovery of Otolithic Function following Streptomycin Treatment in the Rat^a

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The toxic action of chronic administration of streptomycin sulfate (STP) on the vestibular hair cells of mammals is well documented.^{1,3-6} Preliminary findings of our group in pigmented rats described severe alterations of motor abilities but an absence of deleterious effects on semicircular canal function (assessed with postrotatory nystagmus) or auditory function (assessed with evoked auditory potentials) after prolonged treatment with STP. These results suggest that STP specifically disrupts otolith organ function in the rat.^{6,9}

Recently, we described gradual recovery of vestibular biochemistry and function in guinea pigs following chronic treatment with STP.⁷ In a morphological study in guinea pigs treated with gentamicin rather than STP, hair cell stereocilia were regenerated after discontinuation of gentamicin injections.²

Because mature rodents are considered to have ceased production of sensory and neuronal elements, these findings are intriguing, and they encouraged us to investigate further the deleterious effects of STP and the possible mechanism involved in recovery after chronic administration of this antibiotic in the mammalian ear using the pigmented, Long-Evans rat as a model. The aim of our work is (a) to confirm an otolith organ toxicity for STP, (b) to identify the cell type affected, and (c) to assess whether recovery occurs in the pigmented rat.

In this paper we report analysis of swimming behavior and morphology by optical microscopy of the sensory epithelium of the utricle in the pigmented rat during and following STP treatment.

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METHODOLOGY

Treatment Protocol

Twenty-day-old male Long-Evans rats were used in this study. Seventeen animals were injected daily intramuscularly for 48 to 57 days with 400 mg/kg body weight of STP (PISA Laboratories, Mexico) dissolved in physiological saline (SPS). Eleven rats served as controls and received SPS injections for the same time interval and conditions as their experimental comrades. Three of the 57-day-treated rats and three of the SPS-injected animals were used to follow recovery for 8 to 12 weeks and did not receive any STP or SPS beyond the 48th day.

Swimming Analysis

Swimming behavior was assessed at approximately one-week intervals by placing the rats in a water tank at 27°C and recording, on videotape, swimming activity for 45 sec. Analysis and classification of swimming patterns were performed after the test.

TABLE 1. Percentage of Rats Displaying Each of the Disrupted Swimming Patterns

Experimental Condition	Swimming Characteristics			
	Vertical Swimming with Roll	Barrel Rolling	Corkscrew Swimming	Forward/Backward Looping
48 Days of Treatment	90	60	80	40
8 Weeks Post Treatment	100	0	33	0

Morphology

After completion of each experimental manipulation, two of the 48-day-treated rats and two of the treated and allowed to recover animals, plus two of the SPS injected rats were deeply anesthetized and transcardially perfused with aldehyde fixative. The auditory bullae were extracted and postfixed in 1% osmium tetroxide, dehydrated, and embedded in Araldite. Vestibular organs and half turns of cochlear duct were sectioned at 1- μ m thickness, stained with methylene blue and azure II, and examined by brightfield microscopy.

RESULTS AND DISCUSSION

Abnormal swimming patterns consisting of vertical swimming with rolls, barrel rolling, corkscrew swimming, and forward and backward looping were observed with varying frequencies in rats treated with STP. None of these responses was observed in any test of control rats. Eight weeks post treatment, vertical swimming with rolls remained in all rats. One of the three rats showed corkscrew swimming, but no rat showed barrel rolling or looping. Hence, partial functional recovery was observed (see TABLE 1).

Histological examination of STP-treated rats revealed that in the utricular macula sensory cells presented fused stereocilia and pyknotic nuclei. In addition, some of these sensory cells were in the process of being extruded from the epithelium

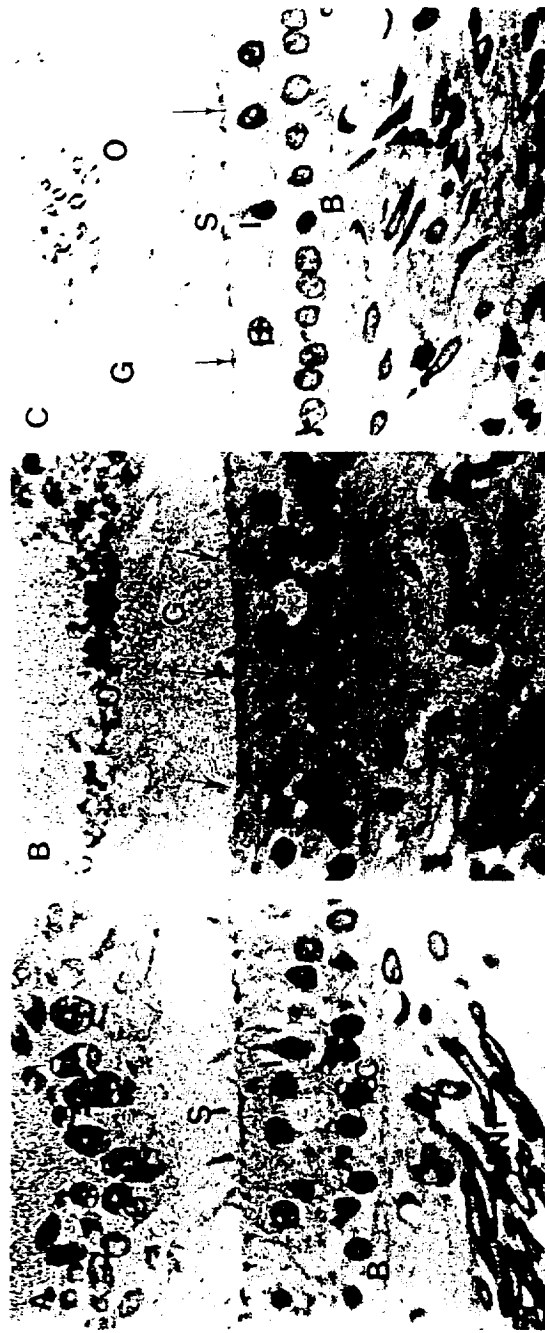


FIGURE 1. One-micron sections of utricular maculae cut perpendicular to endolymphatic surface. (A) Control (saline injected). Hair cell bodies are closely packed together, and stereocilia bundles occur at regular intervals above epithelial surface. (B) Streptomycin treated. Some stereocilia are fused (*big arrows*) and some hair-cell nuclei are pyknotic (*small, thin arrows*). (C) Streptomycin treated and "recovered." Sensory epithelium appears more uniform than in B but slightly atrophic. Hair cells have normal appearance, stereocilia are not fused, but density of bundles is reduced (*arrows*). O, otoconia; G, gelatinous layer of otolithic membrane; I, type I hair cell; B, basal lamina; SC, supporting cells; MNF, myelinated nerve fibers.

(FIG. 1). In contrast, sections of the cristae and organ of Corti appeared normal. In STP-treated and "recovered" animals, neither fused macular hair cell stereocilia nor pyknotic nuclei were observed, but bundle density was reduced. Thus, a partial recovery of sensory epithelium morphology also occurred.

The abnormal swimming behavior observed in rats chronically treated with STP is identical to that observed in congenitally otolith-deficient mice and supports our postulation of otolith organ-specific toxicity of STP in the rat. This is confirmed by our observation that degeneration of hair cells is restricted to the macular organs of antibiotic-treated rats. The partial reversibility of abnormal swimming behavior in animals eight weeks following treatment is in accord with our observations of partial morphological recovery in the same animals. These results show that hair cell and functional recovery can occur in a mammal subjected to prolonged treatment with a clinically relevant toxic agent.

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REFERENCES

1. DUVALL, A. J. & J. WERSALL. 1964. *Acta Otolaryngol. (Stockholm)* 57: 581-598.
2. FORBES, A., L. LI, J. T. CORWIN & G. NEVILL. 1993. *Science* 259: 1616-1619.
3. FUTAKI, T. & I. KAWABATA. 1983. *Adv. Otorhinolaryngol.* 30: 264-267.
4. KROESE, A. B. A. & J. BERCKEN. 1980. *Nature* 283: 395-397.
5. LINDEMAN, H. H. 1969. *Acta Otolaryngol. (Stockholm)* 67: 177-189.
6. MEZA, G., I. LOPEZ, M. A. PAREDES, Y. PENALOZA & A. POBLANO. 1989. *Acta Otolaryngol. (Stockholm)* 107: 406-411.
7. MEZA, G., L. SOLANO-FLORES & A. POBLANO. 1992. *Intl. J. Dev. Neurosci.* 10: 407-411.
8. MEZA, G., N. DAUNTON, L. LOPEZ-GRIEGO & M. SALAS. 1993. *Soc. Neurosci. Abstr.* 19(2): 989.
9. MEZA, G., N. DAUNTON, R. FOX, L. LOPEZ-GRIEGO, H. PRATT & H. ZEPEDA. 1994. *Collegium Otorhinolaryngol. Abstr.* 57.

508.15

THE EFFECT OF GALVANIC TRANSLABYRINTHINE UTRICULAR STIMULATION ON FOS EXPRESSION IN THE GERBIL BRAIN AND SPINAL CORD I.V. Polyakov*¹, G.D. Kaufman², N.G. Daunton¹, R.A. Fox³, and A.A. Perachio². ¹Gravitational Research Branch, NASA Ames Research Center, Moffett Field, CA, ²Dept. of Otolaryngology, Univ. TX Med. Branch, Galveston, TX and ³San Jose State Univ., San Jose, CA.

Previous research has shown that translabyrinthine galvanic stimulation results in different patterns of Fos expression in central neurons depending on the location of the stimulating electrode in reference to specific vestibular end-organs (Kaufman and Perachio, 94). By directing the stimulus through one of the otolith organs, we attempted to model the effect that might be induced by novel gravito-inertial environments. Under general anesthesia, a silver electrode was placed against thinned bone overlying the utricular macula. The following day, cathodal (excitatory) current (50 μ A) was applied for 30 minutes to alert animals. Animals were perfused 1 hour later. Fos immunoreactivity (Fos-IR) was determined by using the avidin-biotin immunoperoxidase method. Sham surgeries were performed to rule-out non-galvanic Fos expression. In stimulated animals, light microscopic observations revealed Fos-IR cells in asymmetrical patterns in the medial vestibular, prepositus, and inferior olivary nuclei. Lumbar spinal cord sections showed contralateral Fos-IR cells in the dorsal horn laminae, while cervical sections also had central and ventral horn labeling. Fos-IR cells were also observed in layers II, III and IV of the contralateral sensorimotor cortex. These observations might represent vestibulospinal and vestibulocortical plasticity with regard to adaptive changes elicited by altered gravitational inputs. Supported by NASA: NRC Associateship, TASK 199-16-12-01, and NAGW-5064.

Light microscopic image analysis system to quantify immunoreactive terminal area apposed to nerve cells

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Abstract

The present report describes a desktop computer-based method for the quantitative assessment of the area occupied by immunoreactive terminals in close apposition to nerve cells in relation to the perimeter of the cell soma. This method is based on Fast Fourier Transform (FFT) routines incorporated in NIH-Image public domain software. Pyramidal cells of layer V of the somatosensory cortex outlined by GABA immunolabeled terminals were chosen for our analysis. A Leitz Diaplan light microscope was employed for the visualization of the sections. A Sierra Scientific Model 4030 CCD camera was used to capture the images into a Macintosh Centris 650 computer. After preprocessing, filtering was performed on the power spectrum in the frequency domain produced by the FFT operation. An inverse FFT with filter procedure was employed to restore the images to the spatial domain. Pasting of the original image to the transformed one using a Boolean logic operation called 'AND'ing produced an image with the terminals enhanced. This procedure allowed the creation of a binary image using a well-defined threshold of 128. Thus, the terminal area appears in black against a white background. This methodology provides an objective means of measurement of area by counting the total number of pixels occupied by immunoreactive terminals in light microscopic sections in which the difficulties of labeling intensity, size, shape and numerical density of terminals are avoided. © 1997 Elsevier Science B.V.

Keywords: Image analysis; FFT; NIH-image; Quantitative immunocytochemistry; GABA; Somatosensory cortex; Light microscopy

1. Introduction

The quantitative assessment of antibody immunocytochemistry in light microscopic sections presents well known difficulties. These include non linearity of optical density measurements of immunoreactive products, uneven lighting and subjective evaluation of staining intensity. In the course of our research (D'Amelio et al., 1996) we explored the possibility of decreasing subjective bias by using a computer-based image analysis technique to measure the area (in pixels) occupied by

immunoreactive terminals in close apposition to nerve cells. Other approaches with similar purposes have previously been reported (Vincent et al., 1994).

2. Materials and methods

2.1. Animals, perfusion fixation and sectioning

Sprague–Dawley rats (200–250 g) were employed for this study. The animals were deeply anesthetized with Metophane[®] and immediately perfused via the heart with 50 ml of 0.9% saline, followed by 500 ml of a fixative made up of 1% paraformaldehyde and 2%

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glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed the same day, immersed in fresh fixative and stored at 4°C. The right hemisphere was coronally blocked between Bregma –1.8mm and Bregma –3.6mm, where the somatosensory representation of the hindlimb is conspicuous and associated with the presence of the rostral hippocampus (Paxinos and Watson, 1986). Coronal sections 40µm thick were cut on a Vibratome® and collected in TBS (0.05 M Tris buffer, 0.9% saline, pH 7.6).

2.2. Immunocytochemistry

The tissue sections, both experimental and control, were processed together in the same solutions to minimize labeling differences.

Floating sections were incubated for 5–10 min at room temperature (RT) with 3% hydrogen peroxide in 10% methanol in TBS and subsequently rinsed four times in TBS × 30 min (RT). The sections were then immersed in GABA antiserum (Chemicon, Cat. # AB131) or control serum (preimmune rabbit serum) diluted at 1:1000 in TBS for 48–72 h at 4°C, with orbital agitation. Then, they were rinsed four times in TBS × 30 min (RT) and incubated for 60 min (RT) in swine anti-rabbit IgG diluted 1:50 in TBS. The sections were rinsed four more times in TBS × 30 min (RT) and then incubated for 60 min (RT) with rabbit peroxidase–antiperoxidase complex (Sigma) diluted 1:200 in TBS. To develop reaction product the sections were immersed in 12.5 mg diaminobenzidine tetrahydrochloride (DAB) in 50 ml TBS + 5 µl 30% hydrogen peroxide for 5–8 min. Finally, the sections were rinsed in TBS, two changes × 10 min (RT), mounted on gelatin coated slides, air-dried and coverslipped with Permount®.

2.3. Image analysis equipment

2.3.1. Light microscope

Sections were observed under a light microscope (Leitz Diaplan) equipped with a 100 W halogen lamp and with a Fluotar 100 1.32 oil immersion objective. Two filters (a Kodak Polycontrast photographic filter #1(12) and a Wratten gelatin filter #15, deep yellow) were placed in the microscope light path to enhance contrast and increase accuracy of focus.

2.3.2. Image analysis system

Images were captured using a Sierra Scientific (Sunnyvale, CA) Model 4030 CCD camera. This is a black and white video-rate camera with 640 horizontal scan lines and 492 vertical scan lines. It was mounted on the microscope body connected to a Scion Technology (Friederick, MD) LG-3 frame grabber board installed in a Nubus slot in a Macintosh Centris 650

computer (Cupertino, CA). The LG-3 board samples the analog video signals from the camera into a 640 × 480 grid of pixels with a resolution of eight bits. The brightness level of each pixel ranges from 0 to 255 gray levels as it is converted into the digital image. The public domain software, NIH-Image v. 1.59 (written by Wayne Rasband, NIMH, Bethesda, and updated frequently), was used to capture images and to analyze the GABA-IR terminals. This software is available electronically from the Internet by anonymous FTP from zippy.nih.nih.gov/pub/nih-image/nih-image or from the NIH's Web site (<http://rsb.info.nih.gov/nih-image>).

2.4. Image processing steps

2.4.1. Image capture

Our analysis was focused on GABA-immunoreactive (GABA-IR) terminals closely apposed to pyramidal cells of layer V of the somatosensory cortex. Pyramidal neurons were identified by round or oval contours and a distinct apical dendrite. No GABA-IR product was present in the soma of these cells.

Once a pyramidal cell was selected to be analyzed, the light source intensity for the microscope and the video control menu (gain and offset) under NIH-Image were adjusted until the peak intensity of the gray level displayed from the live histogram was close to the midpoint of the range between 0 and 255. Then, the microscope stage was moved off the tissue without changing any video control settings, and a blank field was captured. The latter was stored in the temporary memory of the system. Subsequent cell images, under software control, were captured 16 times and then were averaged to reduce random electronic noise originated from various sources including the camera's CCD sensors, frame grabber, and monitor (Inoué, 1986). The software automatically subtracted the blank field from the averaged images before the final images were captured by the frame grabber to further improve the signal to noise ratio. Thus, GABA-IR terminals in the captured images appeared to stand out better and random noise was reduced. To maximize use of computer storage space, the final captured images were cropped to the size of each pyramidal cell, usually at least 260 × 300 (horizontal × vertical) pixels in size, and saved. Further image processing and analysis was performed on those cropped images. Neurons from both control and experimental sections were captured without changing light and video settings.

A light microscopic microphotograph under oil immersion depicts pyramidal cells outlined by GABA-IR terminals (Fig. 1A). Fig. 1B shows the captured and cropped digital image of one cell.

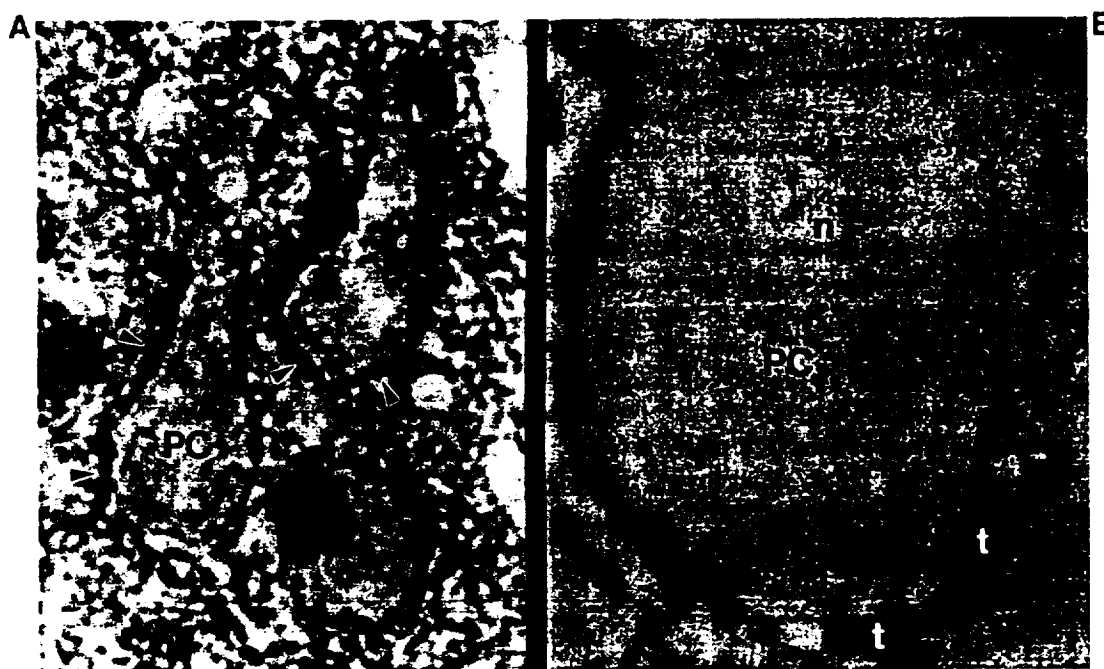


Fig. 1. (A) Microphotograph of pyramidal cells (PC) outlined by GABA-IR terminals (arrowheads) in layer V of the hindlimb representation of the rat somatosensory cortex. Magnification is $600\times$. (B) Cropped digital square image of a pyramidal cell soma (PC) outlined by GABA-IR terminals (t) captured from a microscopic slide viewed under $100\times$ oil immersion objective; n, nucleolus.

2.4.2. Preprocessing of the digital image

Under the Process menu in the software, a type of neighborhood ranking operation—median filter with a 3×3 pixel matrix—was used to reduce electronic noise in the captured image. This filter sorts the nine pixels in each 3×3 neighboring region and replaces each center pixel from the source image by the median value of its eight neighbors. The effect is to remove all pixels that are darker or brighter than their neighbors, and thus remove noise. This is a linear filter operation in which no information is lost from the original image (Russ, 1994). Following median filtering, a sharpening process (also under the Process menu) to enhance the boundaries of terminals was applied.

Fig. 2 shows a flow chart of the image processing steps.

2.5. Image analysis steps

2.5.1. Fast Fourier Transform (FFT)

Fourier Transform (FFT) routines were employed to analyze immunoreactive terminals outlining pyramidal cells in layer V of the hindlimb representation of the somatosensory cortex. The algorithm used in NIH-Image v.1.59 and subsequent versions uses the computationally advantageous Fast Hartley Transform or FHT, (Bracewell, 1986), a close relative of the well known Fast Fourier Transform. The FHT was originally implemented by Arlo Reeves (1990) in his spin-off version of Image FFT. These routines were written in assembly

language specific for the 68000 processor for v.1.28 of NIH-Image. They have now been adapted to current chip technology in v.1.59.

2.5.2. FFT macros

FFT macros are invoked initially using 'Load macros' under the Special menu. A square area, comprised of 128×128 or 256×256 pixels (the size of this selected area must be a power of two, a requirement of the FFT), was selected by applying one of the procedures in the FFT macros. The FFT was performed on a square area of the image to obtain a power spectrum image in the frequency domain (Fig. 3). Different spatial signals from the original image were represented as different frequencies at various distances from the center of the power spectrum, with concentration of lowest frequencies closer to the center, and the higher frequencies further away from the center.

2.5.3. Inverse FFT

A software filter (under FFT macros in the Special menu), size 80% (retaining 80% of the original frequencies), and a transition zone, with a width of 20%, was created and applied to all cells for analysis (screen display in Fig. 4). When an inverse FFT with filter procedure (under FFT macros) was employed, the information in the frequency domain is transformed back to the spatial domain (screen display in Figs. 4 and 5). This operation restores the original image with the high frequencies suppressed, making the image of the terminal area more prominent.

2.5.4. Boolean 'AND'ing and thresholding

In this step the original image was pasted to the transformed image using the Boolean logic operation 'AND' (under 'Paste control' option in Windows menu), so that the terminals in focus were clearly delineated from the background (screen display in Figs. 6 and 7). If a cell was larger than the 256×256 area, a composite of squares was made, using Boolean logic 'OR' under 'Paste control' to match the squares. As to the threshold, instead of having to adjust it according to each individual image, in this application the threshold end point was set at 128 for all images. This end point consistently selected all pixels of the terminals, whereas in settings beyond 128 many pixels would remain unselected. A binary image was then

Image Processing Steps

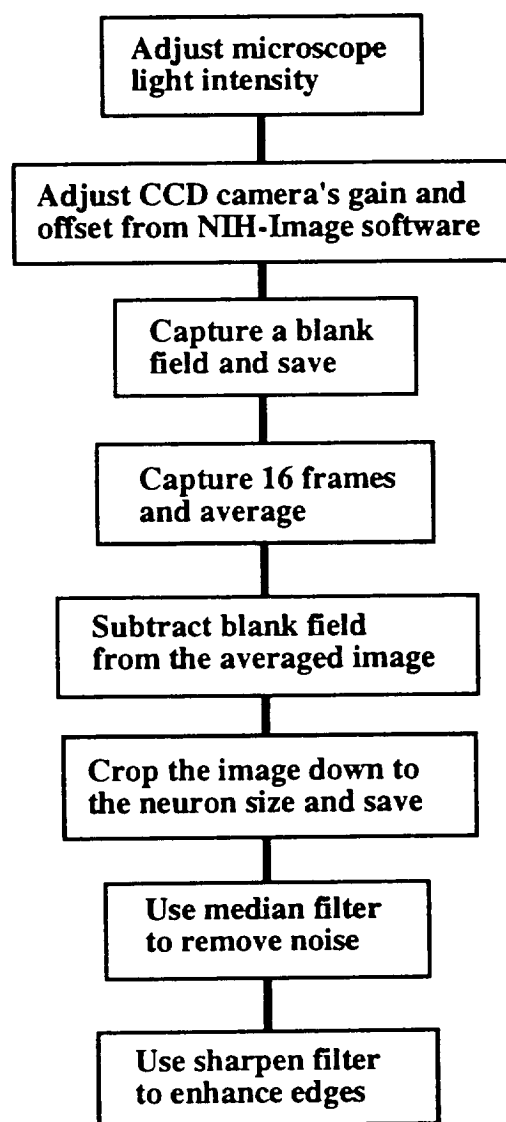


Fig. 2. Flow chart of the image processing steps.

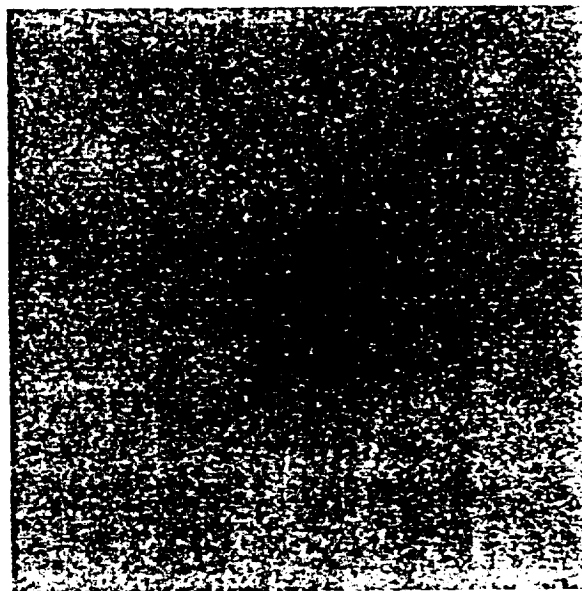


Fig. 3. Power spectrum of a pyramidal cell produced by FFT of a square image in the frequency domain.

created that revealed the terminal area in black against a white background (Fig. 8).

2.5.5. Measurements

The terminal area was measured on binary images and the perimeter of the cell bodies was estimated on gray scale images. The PENCIL tool in the software was employed to separate the clearly delineated terminals in close apposition to the pyramidal cell from those axon terminals that were not apposed (Fig. 8). To measure the area of the terminal, the WAND tool, an automatic measuring tool (highlighted in Tools window in Fig. 8), was employed to count the number of pixels in the black zone, when the area measurement option in the software was selected. With the shift key depressed, individual measurements were added together. The perimeter of the pyramidal cell body was estimated by using the POLYGON tool to trace the outline of the soma (Fig. 9). The dendritic gaps (apical and basal) were subtracted from the perimeter measurement.

A flow chart of the image analysis steps is shown in Fig. 10.

2.6. Statistical analysis

All measurements were exported directly into Excel (Microsoft, Redmond, WA) for easy record keeping, and for easy computation of the ratio of the area of the terminal to the perimeter of the soma. The ratios from all the cells analyzed were then exported into Super-ANOVA (Abacus, Berkeley, CA) and a one-factor ANOVA was used to evaluate the effect of different experimental conditions on the area occupied by GABA-IR terminals apposed to pyramidal cells.

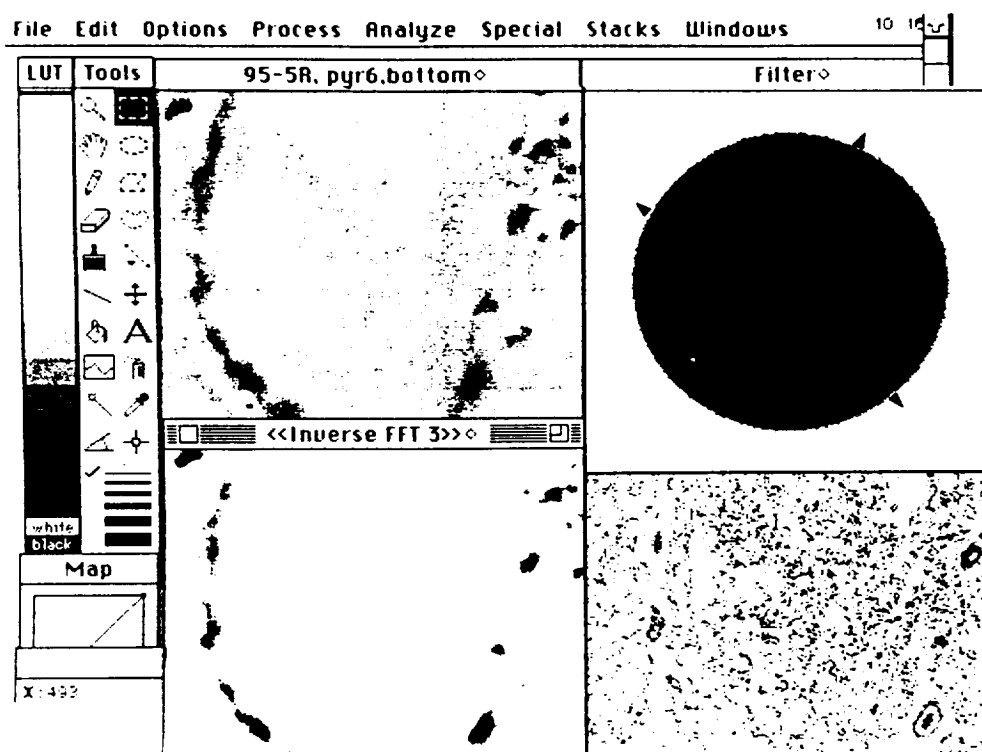


Fig. 4. Screen display of NIH-image software. Upper left corner: square image of a pyramidal cell; upper right corner: filter [size: 80% in black; 20% transition width (arrowheads)]; lower left corner: pyramidal cell soma outlined by GABA-IR terminals after inverse FFT with filter; lower right corner: resulting image from Boolean 'AND' ing the two images from the left side of the screen.

3. Example of data analysis

As part of an ongoing project in our laboratory, the procedure that has been described was employed to analyze the area occupied by GABA-immunoreac-

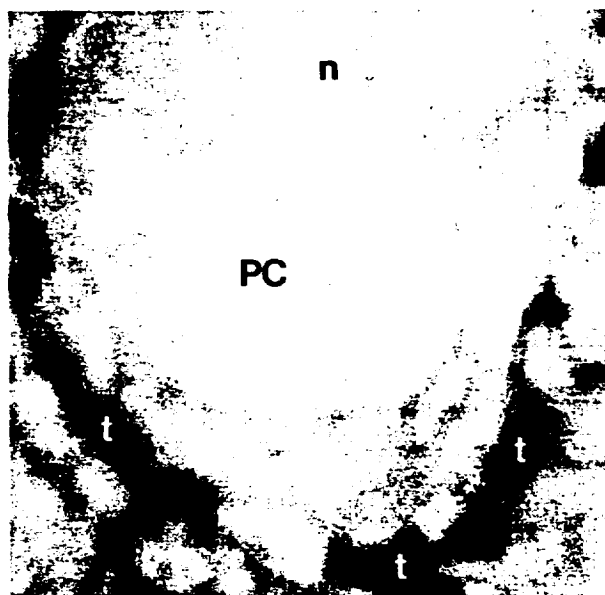


Fig. 5. Resulting image from applying inverse FFT with filter (size: 80%, transition width 20%) restoring the power spectrum image to the spatial domain. PC, pyramidal cell; t, terminals; n, nucleolus.

tive terminals apposed to pyramidal cells in layer V of the hindlimb representation of the rat somatosensory cortex following 14-day exposures to chronic hypergravity (3 G) produced by centrifugation. A significant reduction in the GABA-immunolabeled terminal area was found with respect to the control group. A total of 100 pyramidal cells, each from the control group and from the rats exposed to hypergravity were analyzed. The ratio of the area of GABA-IR terminals to perimeter of pyramidal cell soma was 8.122 ± 0.259 (mean \pm S.E.M.) for the control and 5.008 ± 0.206 for the hypergravity group ($P < 0.0001$). These results demonstrate that the method is effective in determining quantitative differences in immunoreactive terminals (Fig. 11).

4. Discussion

The FFT applied to two-dimensional images is useful for various purposes, such as removing noise for image restoration, finding the periodicity in biological specimens, or for image enhancement to remove motion blur. For example, Russ (1994) has pointed out that a typical image analyst often avoids analysis in the frequency domain because of difficulties in relating the questions to be asked to the problems encountered in image analysis procedures. But with modern day soft-

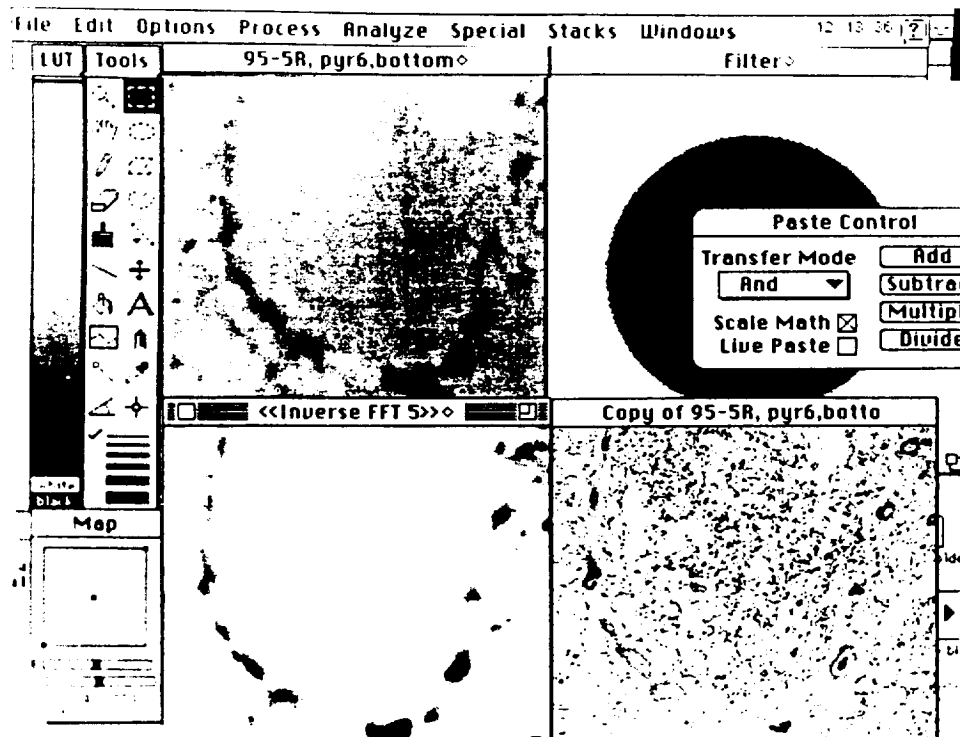


Fig. 6. Screen display showing paste control window with Boolean 'AND' selected. The rest of the screen is similar to Fig. 4.

were such as NIH-Image 'one does not need to deal deeply with the mathematics to arrive at a practical working knowledge of these techniques' (Russ, 1994;

$\beta = 283$). An important property of FFT is that it can be reversed. The inverse FFT applied to the 'forward' FFT, i.e., the power spectrum of an image, restores the original image. Filtering in the frequency domain before applying the inverse FFT, such as we did with the

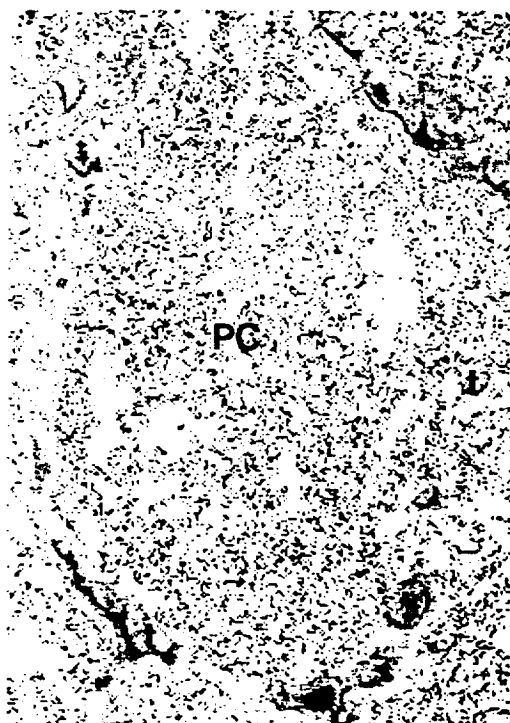


Fig. 7. The resulting image from Boolean 'AND'ing the square image (Fig. 1B) to the inverse FFT image (Fig. 5). GABA-IR terminals are clearly seen in the foreground. PC, pyramidal cell soma; t, terminals.

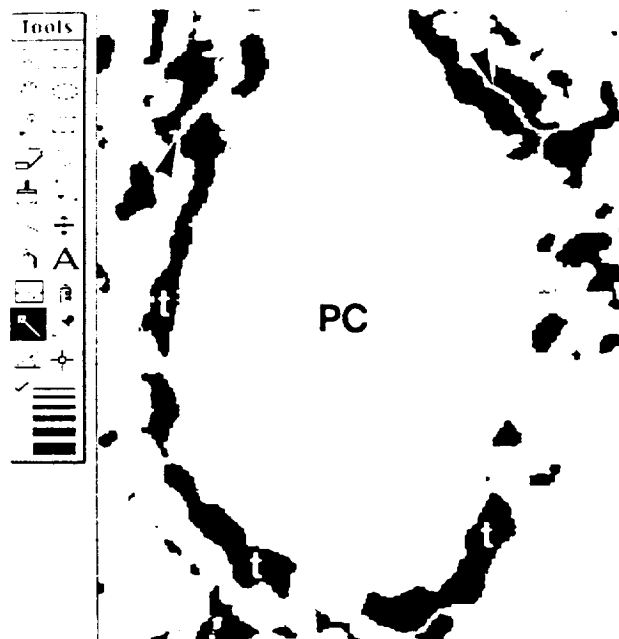


Fig. 8. Binary image after thresholding at 128 showing terminal area in black apposed to a pyramidal cell in white. Axon terminals not apposed have already been separated (arrowheads). The WAND tool is highlighted in Tools window. PC, pyramidal cell soma; t, terminals.

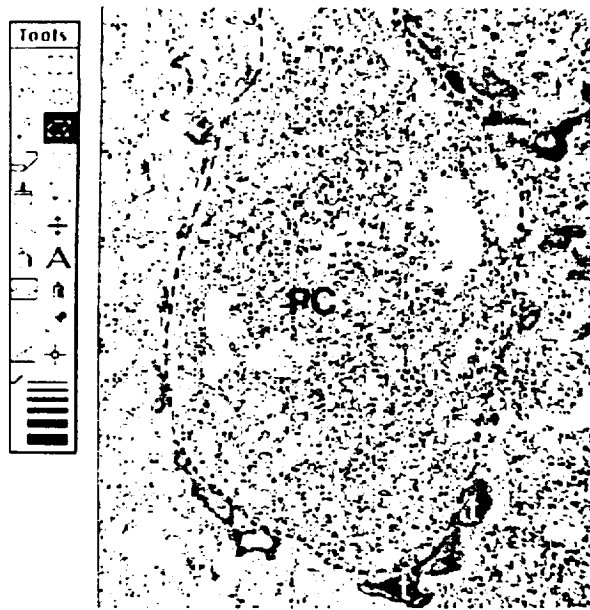


Fig. 9. Gray scale image of pyramidal cell soma (PC) with perimeter outlined employing the POLYGON tool (highlighted in Tools window); t, terminals.

creation of the filter in the image analysis steps, removes most of the noise at higher frequencies. The analysis of images in the frequency domain (by application of FFT), was more efficient than processing in the spatial domain. The latter would have required many different steps of filtering and image mathematics with less satisfactory results. Using the inverse FFT with filtering, and then using the Boolean logic operation, 'AND'ing from the original image to the transformed image is essentially adding the signals together, thus bringing out the terminals into the focal plane and clearly differentiating them from the background. Furthermore, there is a definite endpoint of threshold at 128 that avoids subjective manipulation of threshold that could affect the results. Thresholding is a common step applied to the digital image before measuring. The traditional approach is to define a range of brightness values in the original image so that all the pixels within this range are selected as belonging to the foreground and measured, while all the other pixels belonging to the background are rejected. Since for practical reasons a large number of cells are analyzed over a period of several days, image capture from individual sections is often performed on different days. Thus, changes in lighting conditions may occur. However, after the FFT procedure is applied, a uniform threshold set at 128 can be employed for each cell from any section regardless of variations in lighting conditions.

We believe that the procedure described in the present report is useful since it increases the accuracy of the analysis by decreasing subjective interpretation and avoiding the difficulties and shortcomings presented by,

for example, the quantitative evaluation of optical density in samples stained with immunocytochemical methods. These include variations in labeling intensity, the need for a strict control of antibody concentration and times of incubation, the possibility of tissue alterations such as the compression of labeled profiles into smaller

Image Analysis Steps

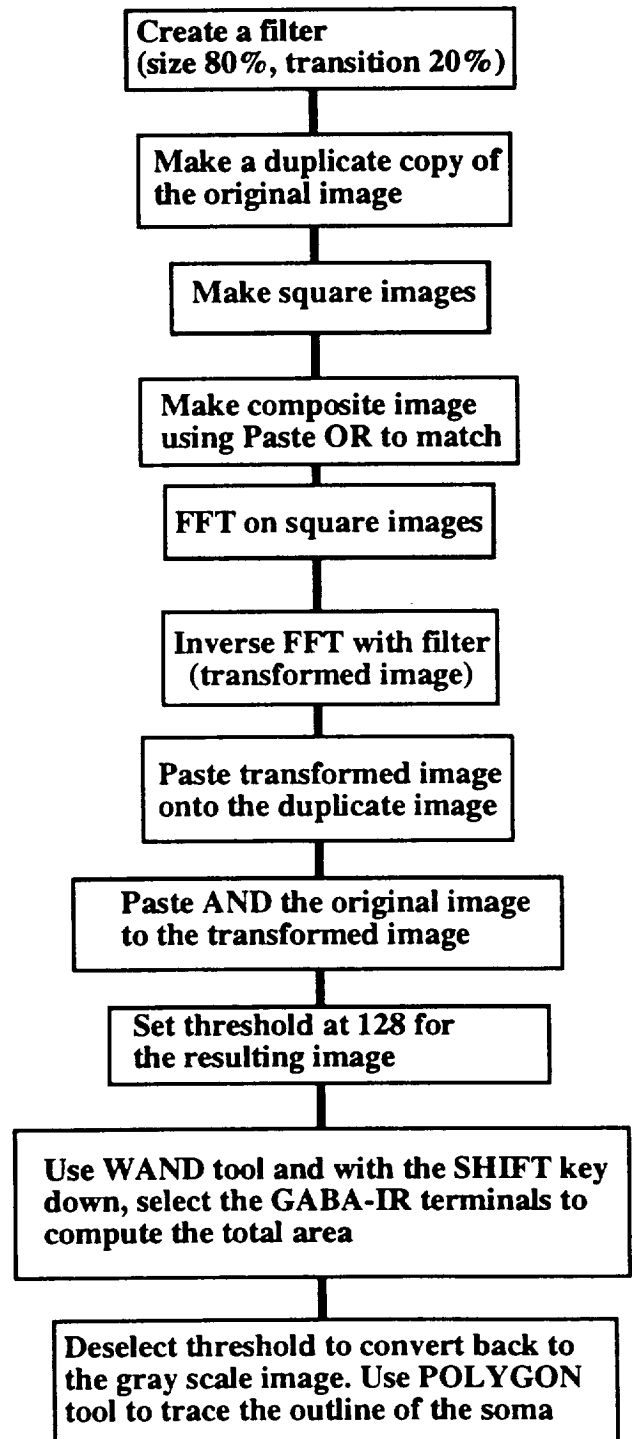


Fig. 10. Flow chart of the image analysis steps.

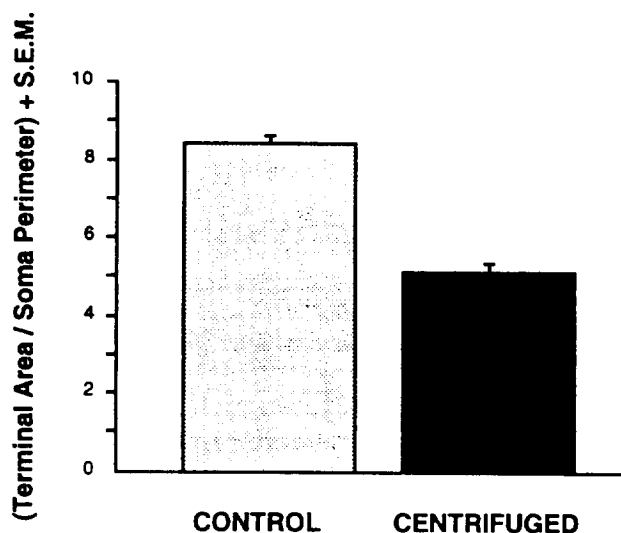


Fig. 11. GABA-immunoreactivity expressed as the ratio of terminal area to perimeter of the pyramidal cell body for animals in the control and centrifuged groups.

areas that may result in erroneous determinations of density and the use of standards containing a known amount of antigen, since differences in optical density may not reflect changes in the concentration of the antigen (Mize, 1989, 1994). These drawbacks are further confounded by the possibility of uneven lighting during image capturing and monitor display that, with our procedure, are less important variables. The same can be said of labeling intensity of immunoreactive products. Although all steps of the immunocytochemical staining for both control and experimental sections were performed in the same solutions to avoid variations in labeling intensity, our procedure allows for such variations to occur without significantly impacting the results. As many researchers have learned, optical density measurements on immunoperoxidase products by means of light microscopy requires a sophisticated image analysis system. To perform those measurements, gray scale images have to be converted into binary images through thresholding. Thresholding that would be optimal for one area, would invariably be unsatisfactory for other areas. Thus, some of the needed features might be rejected or many of the background pixels might be included leading to erroneous result.

In the final analysis, the methodology to be employed should depend upon the questions to be answered. In our research we are interested in determining differences in the area occupied by GABA immunolabeled terminals apposed to pyramidal cells in regions of the

somatosensory cortex (e.g., hindlimb representation) related to proprioceptive inputs from muscles. In this case we believe that, for example, the concentration of the antigen, important for the quantitative assessment of optical density, is less significant. More relevant to our purposes, if differences in GABA immunoreactivity are found between control and experimental samples, is to search for alterations in the synthetic activity of the transmitter.

Finally, we wish to emphasize some of the advantages of our procedure. They include the use of a common desktop computer, relatively inexpensive equipment, readily available free software to attain quantitative analysis, a standard procedure that can be easily followed, and minimal training requirements. (Expert help is also available from the NIH-Image, e-mail group located in soils.umm.edu). The methods described in this paper should well serve the purposes of others attempting to answer scientific questions of a similar nature.

Acknowledgements

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References

- Bracewell, R.N. (1986) The Hartley Transform. Oxford, New York.
- D'Amelio, F., Fox, R.A., Wu, L.C., and Dauntun, N.G. (1996) Quantitative changes of GABA-immunoreactive cells in the hindlimb representation of the rat somatosensory cortex after 14 day hindlimb unloading by tail suspension. *J. Neurosci. Res.* 44: 532–539.
- Inoué, S. (1986) Video Microscopy, Plenum, New York.
- Mize, R.R. (1989) The analysis of immunocytochemical data. In J.J. Capowski (Ed.), *Computer Techniques in Neuroanatomy*, Plenum, New York, pp. 333–372.
- Mize, R.R. (1994) Quantitative image analysis for immunocytochemistry and in situ hybridization. *J. Neurosci. Methods* 54: 219–237.
- Reeves, A.A. (1990) Optimized Fast Hartley Transform with applications in image processing, Thesis, Dartmouth University.
- Russ, J.D. (1994) *The Image Processing Handbook*, 2nd ed., CRC Press, Boca Raton, pp. 283–284.
- Vincent, S.L., Adamec, E., Sorensen, I. and Benes, F.M. (1994) The effects of chronic haloperidol administration on GABA-immunoreactive axon terminals in rat medial prefrontal cortex. *Synapse* 17: 26–35.
- Paxinos, G. and Watson, C. (1986) *The Rat Brain in Stereotaxic Coordinates*, Academic Press, New York.

or tissue staining results and in subsequent trouble-shooting. This assay was used to optimize several *in situ* amplification parameters, including $MgCl_2$ concentrations for designated primer sets, bovine serum albumin blocking of the exposed, charged slide surfaces and the temperature and time periods of thermocycling segments.

Measurement of the actual slide temperature during cycling profiles is important for evaluation of the adequacy of program settings for *in situ* amplification. Instruments that allow the slide to reach the selected temperature rapidly will improve the efficiency of *in situ* gene amplification reactions. Eight different thermocycling instruments, designed for use with microscope slides, were programmed with temperature cycling profiles having identical setpoints and soak periods. Actual temperature profiles measured on a microscope slide varied among the instruments dependent upon the instrument's algorithm, internal temperature calibration and the time necessary to attain the set temperature. The ability of each instrument to amplify DNA in solution PCR reactions on a microscope slide was assessed utilizing the assay described above. The DNA amplification efficiencies achieved on different instruments ranged from the generation of no detectable specific product band to the generation of a single product of the expected size. The appearance of nonspecific product and background amplification correlated with intrinsically long cooling time and less-than-optimal annealing temperatures. Elimination of background bands and improved amplification efficiency on instruments with slow cooling rates could be achieved by simply shortening the soak times. This resulted in an overall shortened cycle time and led to amplification efficiencies comparable to those achieved with faster machines.

Several of the characteristics of each machine that are relevant to thermocycling performance were assessed. These properties are summarized in Table 1.

Affordable Image Analysis System to Quantify Immunoreactive Terminals in the Somatosensory Cortex

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Quantification of immunoreactive product is difficult because many methods of analysis rely on subjective rating. The present report describes an affordable and easy-to-use image analysis system based on public domain software and common laboratory equipment. We demonstrate this system by quantifying immunoreactive terminals in the somatosensory cortex using 40 μ m-thick coronal sections of the rat brain. Tissue sections from control and treated animals were immunoreacted together (1) with GABA antiserum (Cat. #AB131; Chemicon International, Temecula, CA, USA) and viewed using a light microscope (Leitz Diaplan) equipped with a 100 \times oil immersion objective. Images were captured using a Sierra Scientific (Sunnyvale, CA, USA) Model 430 CCD camera mounted on the microscope body with a Scion Technology (Friederick, MD, USA) LG-3 frame grabber board installed in a Macintosh Centris 650 computer.

As an example, our analysis was focused on pyramidal cells of the Vth layer of the somatosensory cortex that were outlined by GABA-immunoreactive (GABA-IR) terminals. Pyramidal neurons were identified by round or oval contours and a distinct apical dendrite. No GABA-IR product was present in the body of these cells. GABA-IR terminals closely apposed to the pyramidal cells were considered for our analysis.

The public domain software, NIH-Image v.1.59 (written by Wayne Rasband, NIMH, Bethesda, and updated frequently), was used to capture images and to analyze the GABA-IR terminals. This software is available electronically from zippy.nih.gov/pub/nih-image/nih-image or from the NIH Web site (<http://rsb.info.nih.gov/nih-image>). Because many laboratories have the basic components of this system, the most common expense for implementation would be the cost of a frame grabber board (typically less than \$1000) and cables to interconnect the camera, computer and associated peripherals.

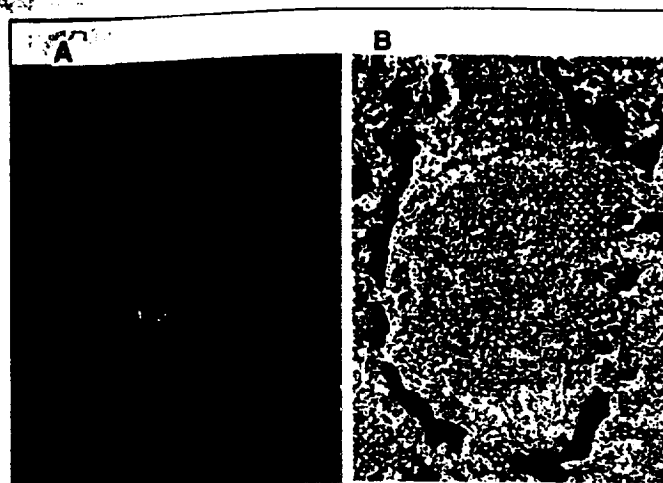


Figure 1. (A) An image of a pyramidal cell outlined by GABA-IR terminals. (B) The resulting image from ANDing the original image to the transformed image. Arrows point to contours GABA-IR terminals.

Starting with v.1.59, NIH-Image has incorporated the Fast Fourier Transform (FFT) routines that were written by Arlo Reeves (Dartmouth University). These routines were written in assembly language specific for the 68000 processor for v.1.28 of NIH-Image and have been adapted to current chip technology in v.1.59. We used these FFT routines to analyze immunoreactive terminals outlining pyramidal cells in the Vth layer of the hindlimb representation of the somatosensory cortex and to compare changes after experimental manipulations. A pyramidal cell outlined by GABA-IR terminals is shown in Figure 1A. A type of neighborhood ranking operation—median filter with a 3 \times 3 pixel matrix—was used to reduce electronic noise in the captured image. After applying the sharpening process to enhance the boundary of terminals, a square area, comprised of 128 \times 128 or 256 \times 256 pixels (the size of this selected area must be a power of 2), was selected by applying one of the procedures in the FFT macro. The FFT was performed on the selected area to obtain the power spectrum (frequency domain). The inverse FFT with filter (size 80%, transition size 20% in our case) was then used to transform back to the spatial domain. Next, the original image was pasted to the transformed image using boolean "AND", so that the terminals in focus were clearly delineated from the background (see Figure 1B). If a cell was larger than 256 \times 256 area, a composite of squares was made. Finally, binary image was created using the *THRESHOLD* operation set at 128.

The area of terminals was measured on binary images and the perimeter of the cell bodies was measured on gray scale images. To measure area, the *WAND* tool (with the shift key pressed) was used to select multiple terminals and the total area of the terminals was computed automatically. Perimeter was measured by using the *POLYGON* tool to trace the outline of the soma.

The ratio of the terminal area to perimeter of the soma was selected to compare cells from the control and the experimental groups. All measurements were exported directly into Excel (Microsoft, Redmond, WA, USA) and changes in ratios from animals exposed to different experimental conditions were analyzed by ANOVA (SuperANOVA; Abacus, Berkeley, CA, USA). No attempt was made to "count" the number of terminals as done by Vincent et al. (2) who also cut their sections at 40 μ m, because their procedure seems highly subjective (due to thickness of sections) and laborious. The procedure described here is efficient, and we believe that it increases the accuracy of the analysis by decreasing subjective evaluation. (Supported by NASA Grants NAGW-4480 and NCC 2-723 to San Jose State University Foundation).

REFERENCES

1. D'Amelio, F., R.A. Fox, L.C. Wu and N.G. Daunton. 1996. Quantitative changes of GABA-immunoreactive cells in the hindlimb representation of the rat somatosensory cortex after 14-day hindlimb unloading by tail suspension. *J. Neurosci. Res.* (In press).
2. Vincent, S.L., E. Adamec, I. Sorensen and F.M. Benes. 1994. The effects of chronic haloperidol administration on GABA-immunoreactive axon terminals in rat medial prefrontal cortex. *Synapse* 17:26-35.